The human B cell response to a pneumococcal conjugate vaccine.

Thesis

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The human B cell response to a pneumococcal conjugate vaccine.

Thesis submitted for the degree of Doctor of Philosophy

By

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For Dad

Abstract

The heptavalent pneumococcal polysaccharide-CRM197 conjugate vaccine (Pnc7) has been introduced to target the major disease causing pneumococcal serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) in childhood where invasive pneumococcal disease remains a significant global cause of childhood mortality. The aim of this thesis was to address questions related to the pneumococcal capsular polysaccharide specific B cell response.

One week after a single dose of Pnc7 vaccine it was possible to detect spontaneously secreting IgG plasma cells in the peripheral blood of 12 month old toddlers and young adults. The plasma cells were identified as either $CD20^{+}CD27^{+}sIg^{+}CD38^{+}$ or $CD20^{-}CD27^{+}sIg^{lo/-}CD38^{+}$.

Prior to immunisation memory B cells specific for pneumococcal capsular polysaccharides were present in 100% of young adults, but barely detectable in adults aged 47-65 years and were undetectable in toddlers. A single dose of Pnc7 induced elevated frequencies of IgG memory B cells in all age groups, but two doses were required in order to generate equivalent frequencies in adults and toddlers. The generation of IgG memory was dependent on age at time of immunisation and on the serotype polysaccharide.

Generation of IgG memory requires previous natural exposure or immunisation to prime the immune system. Such exposure generates IgM memory in young children and enhancing IgM memory may be important for protection. The IgM response can be replaced by generation of a high avidity IgG response, enhance protection of the very young and elderly through immunisation.

Declaration

I would like to declare that this thesis was completed by me and that all the work carried out personally unless otherwise stated in the acknowlegements. All sources of information are acknowleged by means of reference.

Acknowledgements

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I love you all, thanks.

List of publications

Papers

Clutterbuck EA, Oh S, Hamaluba M, Westcar S, Beverley PC, Pollard AJ.
Serotype specific and age dependent generation of pneumococcal polysaccharide specific memory B cells and antibody in response to immunization with a pneumococcal conjugate vaccine. Clin Vaccine Immunol. 2008 Feb;15(2):182-93. Epub 2007 Nov 21.

Clutterbuck, EA, Penny Salt, Sarah Oh, Arnaud Marchant, Peter Beverley, Andrew John Pollard. The kinetics and phenotype of the human B cell response following immunization with a heptavalent pneumococcal-CRM197 conjugate vaccine. Immunology. 2006 Nov;**119**(3):328-37.

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Clutterbuck, EA, Rajeka Lazarus, Mainga Hamaluba, Peter C. Beverley, Andrew J Pollard.
Determination of the memory B cell response to pneumococcal immunisation is dependent on the *in vitro* stimulants used for differentiation of B cells into antibody forming cells. Poster presentation at the 6th ISPPD conference, Rekjavik, June 2008.

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The *in vitro* proliferative response of peripheral blood B cell subsets to polyclonal stimuli is reduced in elderly compared to young adults. Poster presentation at the 6th ISPPD conference, Rekjavik, June 2008.

Clutterbuck, EA, Oh, S, Hamaluba, M, O'Connor, S, Beverley, P, Pollard, AJ
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The kinetics of the polysaccharide specific plasma cell and memory B-cell response in adults receiving a booster immunisation of heptavalent pneumococcal-CRM_{197} conjugate vaccine. Poster presentation at the 23rd ESPID conference, Valenica 2005.

Clutterbuck, EA., Salt, P., Marchant, A, Moxon, E.R., Beverley, P., Pollard, AJ
The kinetics of pneumococcal polysaccharide specific IgG-antibody secreting cells (ASC) isolated from 12-month old infants following a single dose of a 7-valent pneumococcal-CRM197 (Pnc-7) conjugate vaccine. Poster presentation for the Frontiers in Neonatal and Infant Immunity conference, Madrid, March 2005.

Clutterbuck, E.A., Salt, P., Marchant, A., Moxon, E. R., Beverley, P., Pollard, A. J.
Suppression of spontaneous IFN-γ production by PBMCs isolated from 12 month old infants following vaccination with a 7-valent pneumcoccal CRM197 conjugate vaccine. A poster presentation at the 4th ISPPD conference, Helsinki, May 2004.

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List of abbreviations

Ab	antibody
AFC	antibody forming cell
AID	activation induced cytidine deaminase
AOM	acute otitis media
APC	antigen presenting cell
APRIL	A proliferation inducing ligand
ASC	antibody secreting cell
αβ-T cell	alpha-beta T cell receptor
BAFF	B-lymphocyte stimulation protein
BAFF-R	BAFF receptor
BCR	B cell receptor
BCMA	B cell maturation factor
BlyS	B lymphocyte stimulator
C3b	complement component 3b
CbpA	Choline binding protein A (PspC)
CC	clonal complex
CFSE	(5-(and-6)-Carboxyfluoresceindiacetate succinimidyl ester
CpG	Cytosine-phosphorothioate-guanine
CPS	cell wall polysaccharide
CR	complement receptor
CRM197	mutant diphtheria toxoid-cross reactive material
CRP	C-reactive protein
CSF	cerebro-spinal fluid
CSR	class switch recombination
CwPS	Cell wall polysaccharide
DC	dendritic cell
dip	diphtheria toxoid
DMSO	Dimethylsulfoxoide
DTaP	diphtheria-tetanus-acellular Pertussis vaccine
EDTA	Ethylenediaminetetraacetic acid di-sodium salt dehydrate
Fab	fragment antibody binding
Fc	fragment complement binding
FcR	Fc receptor
FO B cell	follicular B cell
GC	germinal centre
γδ T cell	gamma delta T cell receptor
HABS	Human AB serum
10%HABS	RPMI+HABS
HbOC	Haemophilus B conjugate vaccine
HCl	hydrochloric acid
HiB	*Haemophilus influenzae* type B
HLA	human leukocyte antigen
hsp	heat shock protein
ic	immune complex
iC3b	inactivated C3b
iDC	interdigitating dendritic cell
IFNγ	Interferon gamma
Ig	Immunoglobulin
sIg	surface bound Ig
sIgA	secretory IgA
IL-	Interleukin-
IPD	invasive pneumococcal disease

IPV	inactivated Polio vaccine
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MEF	middle ear fluid
MHC	major histocompatability complex
mHSA	methylated Human serum albumin
MLST	multi locus sequence typing
MZB	marginal zone B cell
NaOH	sodium hydroxide
NBBS	Newborn bovine serum
10%NBBS	RPMI+NBBS
NK cell	natural killer cell
NP	nasopharyngeal
NVT	non-vaccine type
ODN	oligodinulceotide
OMPC	outer membrane protein complex from *Neisseria meningitdis*
OR	Odds ratio
OS	oligosaccharide
PAF-r	platelet activating factor receptor
PAMPs	Pathogen associated molecular patterns
PB	plasmablast
PBS	Phosphate buffered saline
PBS-T	PBS+Tween20
PBMCs	Peripheral blood mononuclear cells
PBP	penicillin binding protein
PC	plasma cell
PCh	phosphorylcholine
PHA	Phytohemagglutinin
pIgR	poly immunoglobulin receptor
PMN	polymorphonuclear leukocyte
PmpA	putative protease maturation protein A
Pnc	pneumococcal conjugate vaccine
Pnc7	heptavalent pneumococcal polysaccharide-CRM197 conjugate vaccine
PRP	polyribosyl ribitol phosphate
PRR	pattern recognition receptor
PsaA	pneumococcal surface adhesin A
PspA	pneumococcal surface protein A
PspC	pneumococcal surface protein C (CbpA)
PVDF	polyvinylidene fluoride
PWM	Pokeweed mitogen
RPMI	(RPMI with L-glutamine and penicillin+streptomycin)
SAC	*Staphylococcus aureus* Cowan strain
SHM	somatic hypermutation
ST	sequence type
TA	teichoic acid
TACI	transmembrane activator and calcium modulator cyclophilin interactor
TBS	Tris buffered saline
TD	Thymus dependent
tet	tetanus toxoid
TFH	follicular T helper cell
TGFβ	transforming growth factor beta
Th	$CD4^+$ T helper cell
Th1	$CD4^+$ T helper cell type 1
Th2	$CD4^+$ T helper cell type 2
TI	Thymus-independent
TI-2	Thymus-independent type 2

TLR	toll like receptor
TNFα	tumour necrosis factor alpha
Treg	regulatory T cell
23PsV	23 valent pneumococcal polysaccharide vaccine
V-gene	variable gene
VT	vaccine type

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Chapter 1: Introduction

1.1 *Streptococcus pneumoniae*: Virulence and pathogenicity factors.

Streptococcus pneumoniae is a commensal organism, harmlessly residing among the natural flora of the nasopharyngeal (NP) mucosa (1, 2). The pneumococcus was first described in 1881 by George Miller Sternberg, who isolated the bacterium from an infected rabbit following prior inoculation with his own saliva (2, 3).

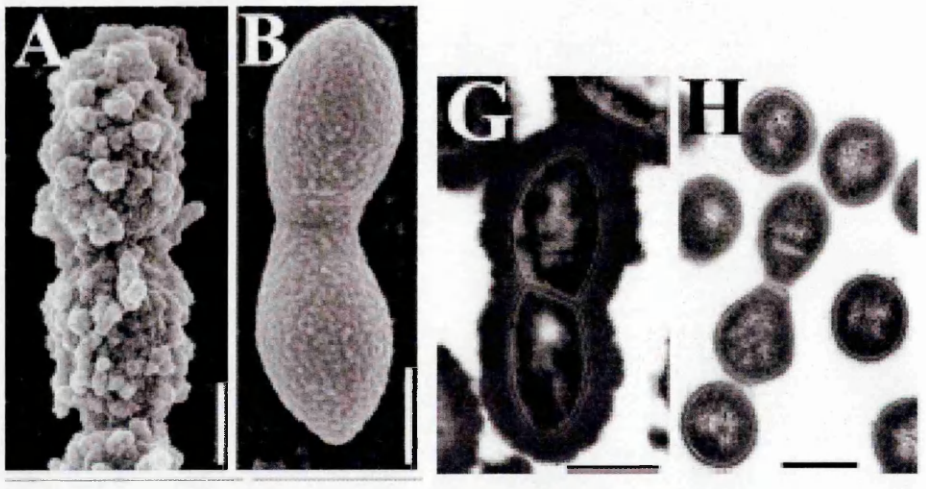


Figure 1.1 An Electronmicrograph of encapsulated (A and G), and non-capsulated (B and H) pneumococci.
Viewed following conventional fixation (A and B), and LRR fixation (G and H) prior to FESEM (Hammerschmidt *et al*, 2005 figure 3 (4)).

S. pneumoniae is a Gram positive bacterium that exists in a characteristic diploid form or as single cells or short, filamentous chains depending on the nutrition available during growth as shown in figure 1.1 (4, 5). The pneumococcus has three major structural outer layers, i) a cytoplasmic membrane, ii) a cell wall and iii) a polysaccharide capsule. Many of the components

of the cell wall and capsule are essential for the virulence or invasiveness of the pneumococcus and the major ones are shown in figures 1.2 and 1.3.

The role of virulence factors includes inhibition of opsonophagocytosis, complement activation and avoidance of specific antibody formation. Pathogenicity results mainly from factors released by the pneumococcus following bacterial cell death and lysis, and the main outcome of these pathogenicity factors is localised inflammation (6, 7).

The pneumococcus is able to exist in both a non-encapsulated (transparent) form and an encapsulated (opaque) form due to phase variation of the polysaccharide capsule (4). The transparent form appears as in figure 1.2, where surface proteins and cell wall components that mediate adherence to host cells are exposed. In this form the pneumococcus is able to adhere directly to the nasopharyngeal mucosa (8). The opaque form, as seen in figure 1.3, expresses the serotype specific capsule that protects the bacterium from both innate and adaptive host immune responses.

1.1.1 Transparent pneumococci

The major antigenic determinants of the pneumococcus are exposed in the transparent phase (figure 1.2). The bacterial **cell wall**, which is in direct contact with the bacterial cell membrane, is composed mainly of a triple layer of **peptidoglycan**. Covalently attached to the peptidoglycan layer are surface proteins and **cell wall polysaccharide (CwPS)**. In the opaque form the peptidoglycan layer also acts as an anchor for the serotype specific polysaccharide capsule of the pneumococcus.

CwPS contains **teichoic acid (TA), lipoteichoic acid (LTA)** and **phosphorylcholine (PCh)** residues that have roles in the direct activation of complement and also in pneumococcal adherence to the epithelial cells of the mucosa (9). The PCh residues promote the adherence of C-reactive protein (CRP) to the pneumococcus (10). CRP is a potent activator of the classical complement pathway that generates C3b that binds to the surface of the pneumococcus leading to

opsonisation. Along with natural antibody to the CwPS the C3b component of complement mediates the clearance of pneumococci from the blood and possibly mucosal surfaces by opsonophagocytic uptake by polymorphonuclear leukocytes (PMNs).

The PCh residues with the surface bound teichoic acid residues contribute to the adherence of pneumococci to host epithelial cells by binding the chemokine receptor platelet activating factor receptor (PAF-r) (9, 11) that is expressed on activated epithelial cells (12). There is evidence from mouse models that infection of the host with influenza virus increases the risk of colonisation and invasive disease caused by the pneumococcus (13). These studies have suggested a mechanism whereby the influenza virus neuraminidase exposes host epithelial cell surface structures and induces production of the inflammatory mediator interleukin (IL)-1 which in turn up-regulates expression of PAF-r, to which the pneumococcus can adhere. This interaction is thought to be among the mechanisms of transit from the mucosa to the blood (12-14). In humans, antibody directed against PCh or teichoic acid residues of the CwPS are generated in response to exposure to pneumococcus (15, 16). However, although some protective influence against invasive disease has been demonstrated in mouse models (15), the same has not been proven in humans (16).

As pathogenicity factors during bacteraemia, both peptidoglycan and CwPS have been shown to induce the inflammation seen in invasive pneumococcal disease (IPD) in mice (5, 12, 17, 18).

Immunogenic surface proteins are also covalently anchored into the cell wall and membrane and the major ones include **pneumococcal surface protein A (PspA), pneumococcal surface adhesin A (PsaA) and pneumococcal surface protein C (PspC, also known as choline binding protein A (CbpA) and SpsA)** shown in figure 1.2 (19).

Of these proteins, PspA in particular is required for full virulence (20, 21) and it is expressed on all pneumococcal serotypes. PspA is attached to the pneumococcal cell wall by a choline binding domain and shows some variability between serotypes. Immunisation with PspA induces cross-protective antibody suggesting a number of common epitopes occur between the different

serotypes (6, 7, 20-22). One reported function of PspA is interference with the alternative complement pathway. It blocks the deposition of the C3 break down product, inactivated C3b (iC3b) on the bacterial cell surface. This allows the pneumococcus to avoid phagocytosis by macrophages that express complement receptors (CR) 2, 3 and CR4 (23).

In humans Factor H binds host cell sialic acid residues and enhances cleavage of C3 convertase (C3bBb). Complement Factor I then cleaves the remaining C3b, preventing it binding to host cell surfaces, thereby preventing opsonisation of host cells Bacterial cell surfaces do not possess the sialic acid residues required for the binding of Factor H, and instead the serum protein, properdin, binds to C3bBb, stabilising it on the bacterial cell surface. This allows alternative complement pathway amplification of C3b and binding to the bacteria (24). Another pneumococcal surface protein, PspC, provides a mechanism that enables pneumococci to interfere with C3b opsonisation process described above. PspC binds Factor H at the bacterial cell surface, this leads to cleavage of the C3bBb complex, thus reducing the deposition of C3b on the bacterial surface (19, 25-30).

PspC binding of Factor H also mediates adherence of pneumococci to the mucosal surface by interacting with the secretory piece of human secretory IgA (sIgA) which then polyimmunoglobulin receptor (pIgR) (19, 25). Deletion of either PsaA or PspC created avirulent mutant strains with reduced ability to adhere to host cell surfaces (7).

Thus expression of PsaA, PspA and PspC by pneumococci counteracts spontaneous deposition of C3b and enhances adherence to the mucosa increasing the length of colonisation time and invasiveness.

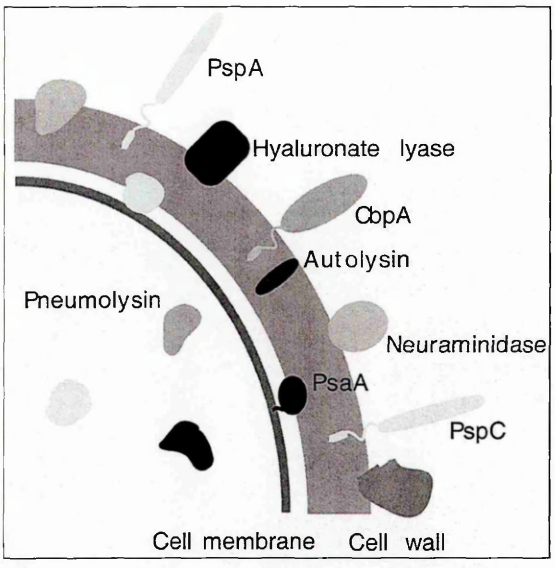


Figure 1.2 A schematic of the non-capsular (transparent) phase of the pneumococcus showing the major virulence and pathogenicity factors (22).

1.1.2 Opaque pneumococci

The major virulence determinant of opaque pneumococci is the polysaccharide **capsule**. It is the capsule that allows identification of pneumococcal isolates, by a test called the Quellung reaction. This process has lead to the identification of over 90 serotypes of encapsulated pneumococci (31). The capsule covers the entire surface of the organism, hiding the immunogenic cell wall and protein components, as shown in figure 1.3, with just PspA extending beyond the cover.

The capsule consists of highly repetitive polysaccharide subunits and in some cases may also contain proteins, glycolipids and PCh residues. In general the presence of the capsule means the pneumococcus is resistant to complement deposition and phagocytosis in the blood (1) but the presence of PCh residues within the capsule structure increase vulnerability to complement deposition mediated by CRP binding to the PCh residues (10). Therefore serotype virulence is determined in part by these differences in the capsule constituents (1). The capsule is produced during the exponential growth phase of the bacteria when conditions are favourable for survival. Encapsulated strains are ten times more virulent than the un-encapsulated strains in mice (32).

The pneumococcus is able to change the serotype specificity of the expressed capsule altering its virulence depending on the underlying genetic strain (33). This was also seen in a mouse model where infection site, capsular type and genetic background all determined virulence of a serotype 3 pneumococcal strain (34).

By regulating the amount of capsule expressed the pneumococcus is able to prolong its survival time in the nasopharynx because the capsule prevents agglutination of the bacteria by the mucous (2, 4, 35). A reduced level of capsule expression, timed with proximity to the epithelia of the nasopharynx and lung promotes adherence mediated by the otherwise occluded surface proteins (4, 35).

The capsular polysaccharides are immunogenic to varying degrees and specific serum antibody is generated following exposure of the host to specific capsular types (1). This serum antibody is crucial in protecting the host against invasive disease (36).

The protective role of the anti-capsular antibody in controlling colonisation is less clear but is aided by antibody directed against the surface proteins, and also $CD4^+$ T-helper cells recruited at the time of infection (37, 38). Murine studies have revealed that the proteins hidden by the capsule are highly conserved and generate less serum antibody than proteins that remain visible to the immune system in encapsulated variants (39). For example the more variable proteins PspA and PspC are detectable by flow cytometry using encapsulated strains, while the more conserved proteins, PsaA and PpmA (putative protease maturation protein A), are not (9, 39). However, data from another mouse study suggested that exposure to pneumococci generates serum antibody specific for conserved proteins and may correlate with protection, while not actually mediating the mechanism of protection (40).

Therefore the capsule not only allows the pneumococcus to survive in the blood stream by occluding highly conserved, immunogenic proteins from the immune system. It also serves to regulate the accessibility of the pneumococcal adhesins to the cell surfaces limiting exposure of

these proteins to the immune system. Variation of the capsular type allows for escape from capsule specific antibody generated during exposure.

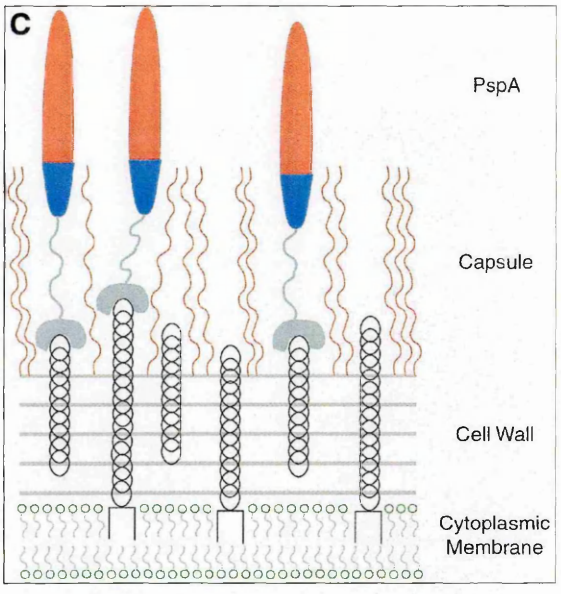


Figure 1.3 A schematic of the encapsulated (opaque) phase variant of the pneumococcus showing the main structural and antigenic components (22).

1.2 The role of secretory components in the pathogenicity and virulence of the pneumococcus.

The pneumococcus secretes a number of enzymes that contribute towards i) bacterial cell adherence to and invasion of the host epithelial cell layer and ii) induction of the inflammatory responses associated with pneumococcal infection.

The pneumococcus must avoid clearance from mucosal cell surfaces in order to cause invasive disease. This is partly achieved by **IgA1 protease**, which acts directly on secretory IgA1 (sIgA1), breaking down the sIgA1 structure, separating the Fc, Fab and IgA secretory component from each other (7). This prevents opsonisation of the pneumococcus and clearance from nasopharyngeal epithelial cell surfaces (41).

Adherence of pneumococci to the surface of host epithelial cells is aided by **neuraminidase**, an enzyme that exists in two forms (**NanA and NanB**). This enzyme cleaves the sialic acid residues on host cell glycolipids (6, 7, 22). Attachment is also thought to be aided by some, as yet unidentified proteins produced by pneumococci that target host cell glycoconjugates containing, N-acetyl-D-glucosamine-β(1-3)galactose, N-acetyl-D-galactosamine-β(1-3)galactose or glucosamine (22). Once the pneumococcus has attached to the epithelial cell surface it may remain for some time or only transiently. The persistence of colonisation may depend on the activity of other bacterial and host cell factors, e.g. teichoic acid residues, CbpA and SpsA. To cause invasive disease the pneumococcus must enter the blood stream. Translocation of the pneumococcus from the epithelial cell surface into the blood is aided by the enzyme **hyaluronidase** which acts on hyaluronic acid present in connective tissue allowing invasion of the bacterium.

The primary mechanism of clearance of pneumococci from the blood is opsonophagocytosis. Capsule specific antibody and the complement component C3b synergise to opsonise intact pneumococci which are targeted for phagocytosis by neutrophils, via FcγRIIa (CD32) and complement receptors (42). Hostetter (43) demonstrated that some capsules blocked C3b to C3d conversion and that this rendered the pneumococcus vulnerable to phagocytosis. Serotypes that enhanced the degradation of C3b to C3d were resistant to phagocytosis and therefore more virulent.

Blood borne pneumococci are also able to avoid opsonisation and phagocytic killing by blocking the binding of complement Factor H and also by **C3-degrading protease** (44). Secretion of **neutrophil esterase inhibitor**, allows the pneumococci to survive within the phagolysosome.

Some of the symptoms of IPD are a result of pathogenicity factors that are released when the bacterial cell is lysed. These pathogenicity factors include the enzyme **autolysin**, which lyses the bacterial cell wall when environmental stress such as penicillin exposure, is encountered and leads to the release of cell wall breakdown products (teichoic acid and peptidoglycan) (22).

Lysis of pneumococci releases **pneumolysin**, an enzyme conserved throughout all pneumococci, from within the bacterial cell and allowing it to lyse host epithelial cells. It has structural homology with CRP and has both lytic and complement activating properties (7). By interacting with Ig-Fc regions it is able to activate the classical complement cascade (45) by binding the PCh residues in CwPS. This leads to complement activation through direct interaction with C1q (a similar mechanism to CRP) (46). Release of pneumolysin from dead bacteria may divert the activated complement cascade away from healthy pneumococci by interacting with C1q molecules at some distance from intact pneumococci (47). The resulting classical complement cascade generates C3a and C5a (anaphylotoxins) that cause vascular permeabilisation, mast cell degranulation and polymorphonuclear cell (PMN) recruitment. Monocytes, attracted to the infection site by the anaphylotoxins, produce tumour necrosis factor-alpha (TNFα) and interleukin (IL)-1β which mediate inflammatory processes. It also inhibits epithelial cell cilia beating and has detrimental effects on neutrophil activity, Ab synthesis and lymphocyte proliferation.

Another function of pneumolysin is the activation of host epithelial cells which release the chemokine, CCL5 which attracts $CD4^+$ memory T cells to the infection site. The cytokines released by these T cells determine the outcome of disease with increased IL-10 and decreased IL-4 and IFNγ inducing transition from carriage to IPD in mice (48).

1.3 Pneumococcal carriage, invasive disease and antibiotic resistance.

1.3.1 Nasopharyngeal carriage

The human host is exposed to pneumococci via transient carriage in the nasopharynx and upper respiratory tract. Carriage is defined as self-limiting colonisation by pneumococci in the absence of lower respiratory tract infection or IPD (49). The incidence of NP carriage increases with age

(50) possibly from birth (51), so that by 2 years of age at least 95% of children have been exposed to one or more episodes of pneumococcal carriage (52). Colonisation rates peak by 3 years of age when up to 50% of children may carry at least one isolate (53) but then decline to reach a stable level of around 8% of children by ten years of age. Pneumococci are then detectable in the nasopharynx at low rates, throughout life providing an important route for community transmission between infants (53) and from infants to adults (54, 55).

Generation of anti-capsular antibody in response to carriage is age and serotype dependent such that in children under 2 years of age carriage is detectable but no antibody is generated while in older children antibody is generated following carriage of immunogenic serotypes but not poor immunogenic serotypes such as 6B (56).

Despite the identification of 90 different serotypes of pneumococcus based on the expressed capsule only a limited number are responsible for invasive disease. The serotypes isolated from the nasopharynx and those obtained from the blood in IPD cases are often similar. For example serotypes 6, 14, 19 and 23 are commonly carried and are also among those responsible for disease in children younger than 24 months of age (50, 57).

The duration of carriage of individual serotypes is related to the age of the host and is affected by the maturity of the immune system (53) and acquisition of carriage peaks during the winter (50). The carriage of pneumococci is thought to be a pre-requisite for the development IPD caused by the same serotype but disease does not occur following every incidence of carriage, even with invasive serotypes (6, 36). In fact it has been reported that invasiveness of a particular serotype is inversely related to the frequency of its detection in the nasopharynx (figure 1.4a (58)) and possibly to the duration of carriage (figure 1.4b (59)).

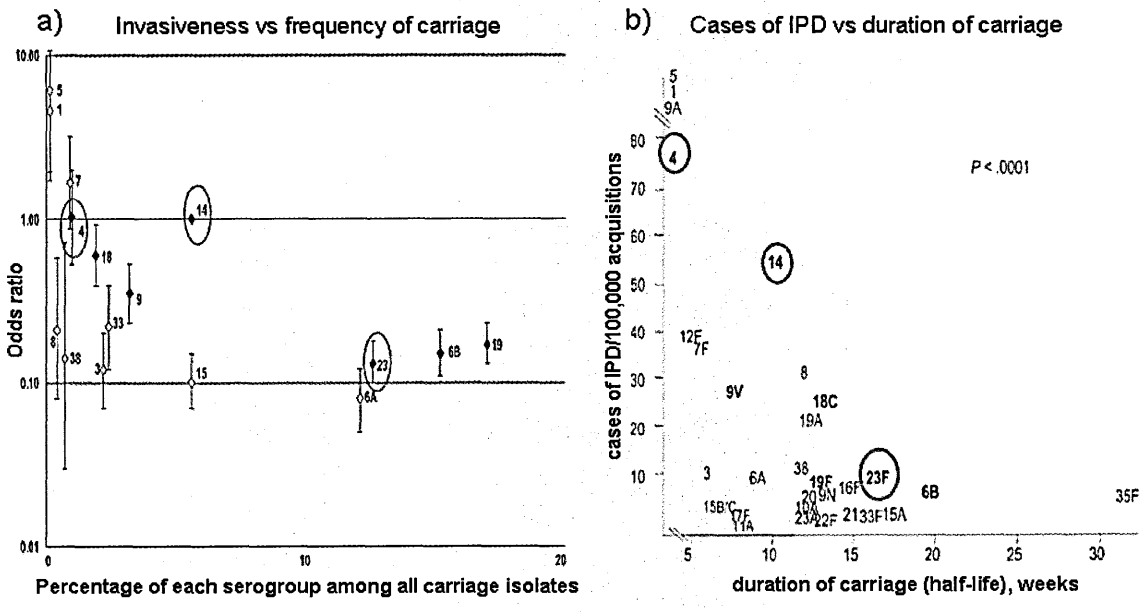


Figure 1.4 The relationships between incidence and duration of NP carriage versus IPD rates.
a) Shows the percentage of serotypes carried compared to the odds ratio (OR) for invasiveness (58). b) Duration of NP carriage versus the number of homologous serotype cases of IPD in the Oxford region (59). The serotypes circled in red are the serotypes investigated in this thesis.

Repeated exposure to pneumococci via the nasopharynx may help to maintain levels of naturally acquired antibody, since antibody levels wane in the absence of carriage of a specific serotype (49, 60). Adults and children over the age of 2 years old generate antibody specific for the polysaccharide capsule and also to the surface proteins in response to NP carriage (56). However the precise role of serotype specific anti-capsular antibody in preventing colonisation is not as clear the role in protection from invasive disease (49). Immune responses that target the surface proteins may also play an important part in the age related decline of carriage and disease rates (4, 37). Antibodies targeting these proteins block adherence to epithelial cells, reducing the duration of carriage (39). Individuals with pre-existing anti-PspA antibodies have been shown to have reduced rates of NP carriage (40) and it has also been suggested that $CD4^+$ T cell help to mediate these anti-protein responses (37, 39).

Prior upper respiratory infections such as influenza can increase the risk of IPD from concurrently carried strains of pneumococcus (14), but by >2 years of age the risk of IPD declines rapidly, but the processes that contribute to this decline are not fully understood (61).

1.3.2 Pneumococcal disease

S. *pneumoniae* is responsible for more than 1 million infant deaths, world wide, per year. It remains the most common cause of invasive bacterial disease in all age groups (37, 62-65). IPD is most common at the extremes of age (<2yrs and >65yrs), where immunological status is either immature or in senescence. Disease occurs despite the universal exposure of individuals to pneumococci via NP carriage. The peak incidence of pneumococcal disease has been shown to occur in the winter months, reaching a maximum rate during January in the USA (66) and the UK (figure 1.10).

The spectrum of infections caused by *Streptococcus pneumoniae* range from localised, mucosal infections, such as acute otitis media (AOM) to severe infections such as pneumonia and blood borne invasive diseases such as septicaemia and meningitis (67).

Data from the Health Protection Agency for 2004-2005 show that the incidence of both meningitis (Figure 1.6a) and all pneumococcal invasive disease (figure 1.6b) peaks during the first two years of life and again in older teenagers and adults, although the incidence of invasive disease is much higher in adults than infants.

Commensal and disease causing serotype occurrence is affected by age, community factors, socioeconomic status and geographical location (63, 64, 68). Scott *et al* (68) found that serotypes 19 and 24 were more frequently carried while serotypes 1 and 5 were often the cause of disease.

They also found that increasing age corresponded with dramatically reduced serotype 14 disease rates and a gradual decline in serotype 6, 18, 19 and 23 disease (68).

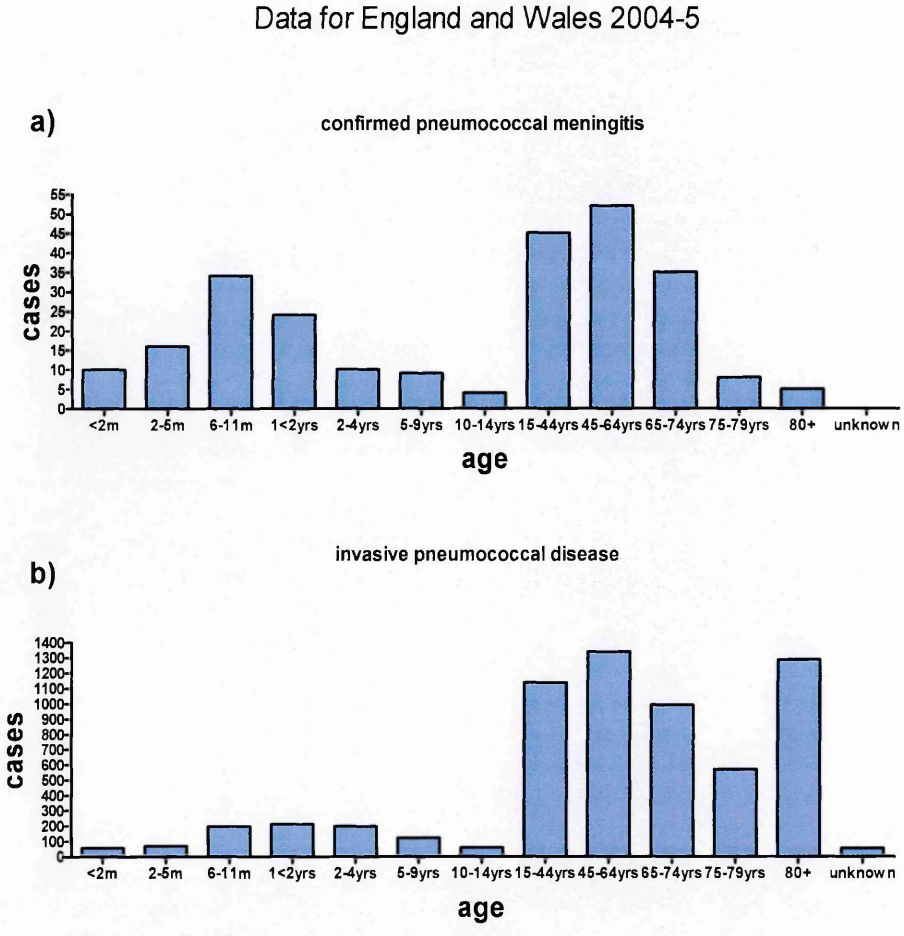


Figure 1.5 Pneumococcal invasive disease and meningitis cases in England and Wales during 2004-5. Data obtained from the HPA, reviewed 10-06-2006.
(http://www.hpa.org.uk/infections/topics_az/pneumococcal/agespecificmeningitiscases.htn and http://www.hpa.org.uk/infections/topics_az/pneumococcal/agespecificlabcasesIPD.htn).

Figure 1.6 shows the effect of geographical location on IPD causing serotype occurrence in the <5 years olds and >5 year olds. Although most disease is caused by serotypes 4, 6, 9, 14, 18, 19 and 23 a significant number of cases are caused by serotypes 1 and 5 in Europe, Africa and Asia but few in the USA and Canada (69).

Anatomical location of disease also affects the serotype recovered. IPD is defined as the isolation of a pneumococcus from a normally sterile site, such as the cerebro-spinal fluid (CSF), middle ear fluid (MEF) or blood. Hausdorff *et al* (70) have reviewed the contribution of different serotypes to disease in these sites and the effects of age and geographical location. Serotype 14 predominated in blood isolates from young children in the USA and Europe while serotype 1 was responsible for most IPD in Asia. In the CSF serotype 6 was most frequently isolated in the USA, Europe, Latin America and Oceania while serotype 2 was found in Asia and serotype 1 in Africa. Data for MEF isolates from the USA and Europe found serotype 19 caused most disease. In older children and adults IPD blood isolates frequently identified serotype 1 in Asia and Europe (70). Hausdorff *et al* (31) also reviewed AOM isolates and found serotypes 19F and 23F were the most common cause of AOM irrespective of geographical location (data from USA, Europe, Argentina and Israel).

Global location also affects the burden of pneumococcal disease with in a population. Parsons & Dockrell (63) reported the incidence of UK IPD to be 6.6/100,1000 (1995-1997), and a similar study determined it to be 37-48/100,000 in the <1 year olds and 21-36/100,000 in the >65year olds (64). USA rates for 1999 were 23/100,000. However, in developing countries IPD rates were much higher with case numbers of 139-224/100,000 in the <2year old age group and 190/100,000 in the adults (71).

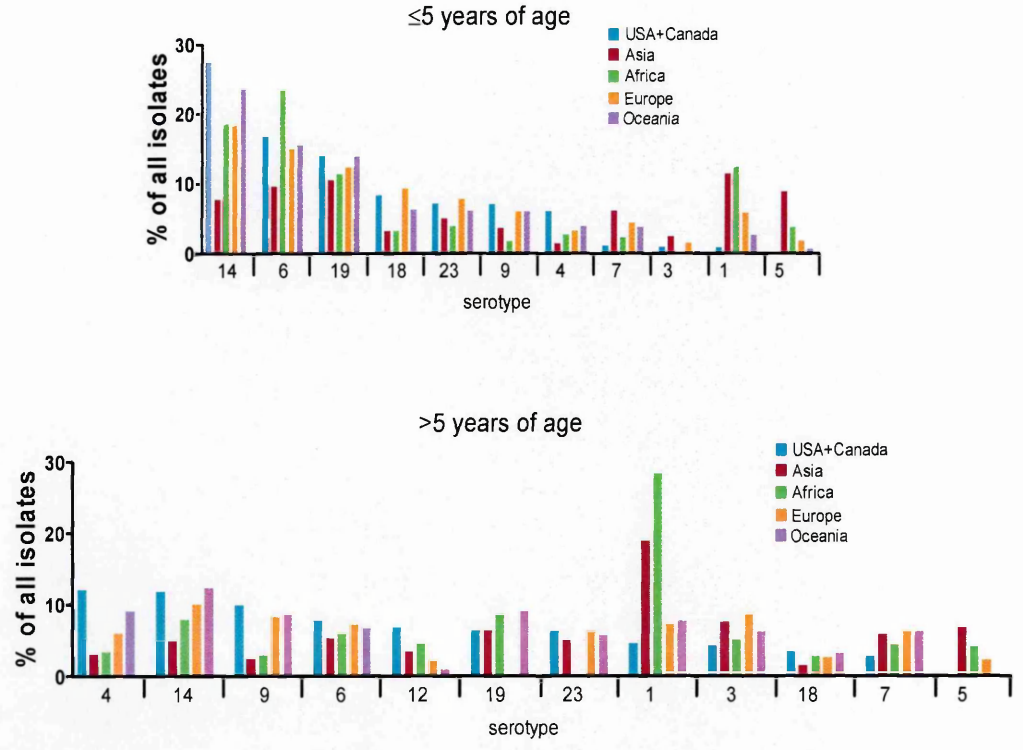


Figure 1.6 Geographical distribution of IPD serotypes. The data represent serotyped isolates as a percentage of all IPD isolates from ≤5 year olds and >5 year olds at five geographical locations. The data are presented in order of decreasing importance in the USA and Canada (blue) plotted with isolate frequency at other locations. Serotypes 1 and 5 are included as important IPD serotypes outside of North America (from table 2 (69)).

A recent UK study (72) found that serotype 14 was most frequently isolated from paediatric IPD cases followed by serotype-1 but some isolates found were not covered by any of the current vaccines. This was also seen in carriage study in Oxford were serotypes 14 and 1 were most commonly isolated from cases in all ages groups combined (figure 1.7). The HPA have shown an increase in the total number of isolates collected in England and Wales with a slight decline in serotype 14 IPD cases (20%-14%), and a rise in serotype 1 cases (4%-13%), for the years 2000/01-2005/06 (www.hpa.or.uk/infections/topics_a2/pneumococcal/serotypeDistribution2000-2006.htm).

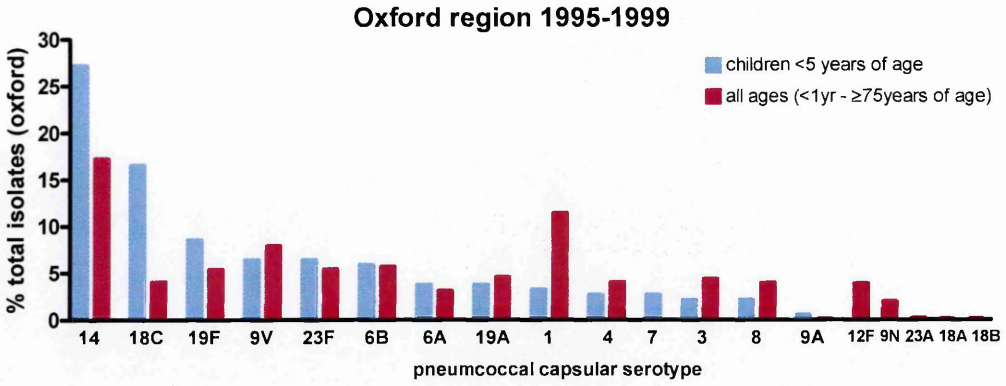


Figure 1.7 Serotypes isolated from IPD cases in the <5 and >5 year olds in the Oxford region (1995-1999).
The data show the percentage of the total isolates, ordered in serotype prevalence in the <5 year old group (blue) with the serotype prevalence for all ages (red). Serotypes not seen in the <5yr olds are at the end of the chart (12F, 9N, 23A, 18A and 18B)(64). Serotypes included in Pnc7 are 4, 6B, 9V, 14, 18C, 19F and 23F.

1.3.3 Antibiotic use and resistance

NP carriage of the pneumococcus combined with high rates of antimicrobial use in industrialised countries is thought to be a major contributing factor to the increase of antibiotic resistance in pneumococcal isolates.

The advent of antimicrobial therapies for infectious disease in the 1930s led to declining numbers of fatal IPD cases (18). Globally, overuse of antimicrobials has led to the generation of pneumococci carrying resistance genes to at least one of the commonly prescribed antibiotics (73). The first described case of penicillin resistance was in 1945 and the first clinically resistant isolates in Australia in 1965, and South Africa in 1977. The serotypes affected appear to be mostly the serotypes associated with infection in children, such as serotypes-6, 14, 19 and 23F (74).

Pneumococci take up genetic elements, such as antibiotic resistance genes from other commensal bacteria, from the environment (75) and incorporate them by recombination events that generate mutations. An example is seen in the gene that encodes the penicillin binding protein (PBP). The

PBP gene is a mosaic gene where recombination events have resulted in reduced affinity of the resulting PBP for penicillin, conferring resistance on the pneumococcus (18, 75). The changes that occur at the DNA level remain within the clonal population even in the absence of antibiotics (75). Development of new drugs such as macrolides, was meant to overcome the increase in resistant pneumococci but replacement of penicillin with macrolides resulted in resistance to this class of antibiotics as well (73). In Canada there has been a 10% rise in resistant isolates since 1997 with β-lactam and macrolide treatment failures reported (73, 75). It was increasing antimicrobial resistance that prompted development of new pneumococcal vaccines with potential for broad protection. Pnc7 vaccine covers most of the common resistant strains (serotypes-6, 9V, 14, 19 and 23) (74, 76, 77) but there are non-vaccine serotypes with resistance to at least one antimicrobial drug, (serotypes-7 and 8), leading to concerns about serotype replacement with non-vaccine types (76, 77).

1.3.4 The relationship between genetic background and the capsule based serotype of the pneumococcus.

Routine pneumococcal typing is based on the expression of the serotype specific, polysaccharide capsule. However, the pneumococcus is capable of exchanging genetic information via horizontal transfer of DNA, including capsule related genes and those coding for antibiotic resistance (78-82). Therefore it became important to understand the genetic relationship between strains traditionally associated with IPD and newly arising strains (78-80, 82). Multilocus sequence typing (MLST) has recently been developed to track invasive pneumococcal isolates by looking at the genetic background of each strain (83). The MLST of an isolate can be related to the occurrence of a particular clone in NP carriage or IPD (83). MLST is based on identifying the alleles of a set of house keeping genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl*) conserved in all pneumococci. A particular set of alleles for each of the seven genes constitutes a sequence type

(ST) (81, 83). Using the relatedness of particular STs different pneumococcal strains have been grouped into clonal complexes (CC) with the parent ST (most common) providing the CC number, (e.g. ST199 belongs to CC199 which also includes closely related ST200, ST667, ST645, ST649 and ST1341) (78). Importantly the particular ST can be associated with multiple serotypes, for example ST199 is commonly isolated expressing serotype 1, 15B, 19A or 19F capsules (84) and ST667 with serotypes 14 and 19 capsules (80). The rate of multiple capsule expression is not known but in one study of 2100 isolates only 11 STs were associated with multiple capsular types (78). The combination of ST and serotype may be related to invasiveness (80) and has been investigated in studies examining the relationship between ST and IPD, AOM or NP carriage (79, 80, 85, 86). A UK study (86) in the <5 year olds determined the ST for 645 pneumococcal isolates. The result was 150 STs associated with 33 serotypes, of which 66% were Pnc11-13 related. Some sequence types occurred in both vaccine type (VT) and non-vaccine type (NVT) serotypes, in particular ST9 was most commonly found expressing a VT serotype 4 capsule but also NVT serotype 8 and 19A capsules (86). Other studies found that IPD strains had less diverse STs than NP strains but particular IPD serotypes may be associated with more than one ST which may alter the invasiveness of the strain. For instance, ST9 was the most prevalent IPD ST in Oxfordshire (79) and was associated with a serotype 14 capsule (Odds Ratio of 8.3) while ST124 with serotype 14 capsule had an odds ratio of 6.4. Thus it was determined that in IPD the capsule had more influence on invasiveness than the ST while it appeared that a combination of ST and capsule aided in the ability of a strain to cause AOM (79, 80, 85).

This association of multiple capsule types with particular STs provides evidence of capsule switching so it was important to determine whether mass immunisation with Pnc7 would place a selective pressure on the occurrence of particular capsule-ST combinations (78, 82). No evidence of exchange of genetic material between VT and NVT strains was found in the <5 year olds but STs occurred in both VT and NVT strains (78). Following the introduction of the Pnc7 vaccine in the USA there was a rise in serotype 19A IPD in the <5 year olds and prior to immunisation

this was associated with CC199. Following immunisation the 19A capsule was also expressed by 6 new STs (82). Of greater concern was the finding of CCs (CC13, CC37, CC81, CC156, CC172, CC199, CC236, CC268, CC1269 and CC1296), that were associated with antibiotic resistance and also with expression of multiple VT serotype capsules (78).

1.4 Pneumococcal vaccines

High rates of IPD treatment failure in the era of antimicrobial therapy due to increasing antimicrobial resistance has generated renewed interest in pneumococcal vaccine development. In 1983 a 23-valent, polysaccharide vaccine (23PsV) was licensed in the USA and then in Europe (87). Subsequently, following on from the success of the *Haemophilus influenzae* type B conjugate vaccines introduced in the USA and Europe in the early 1990s, a heptavalent pneumococcal conjugate vaccine (Pnc7) was introduced in the USA in 2000 and in the UK in the autumn of 2006.

Pneumococcal capsular serotypes included in these vaccines are shown in table 1.1. The relevance of these vaccine serotypes to disease in the UK is shown in figure 1.8. Most cases of pneumococcal infection reported during 2005-2006 were covered by the 23-valent polysaccharide vaccine and seven of the most common serotypes would also be covered by Pnc7 (figure 1.8). The extent of coverage of eleven and thirteen valent conjugate vaccines, currently in trials is also shown. Serotype 5, although not seen in England and Wales during this surveillance period has been among the top 11 or 12 serotypes in the ≤ 5 and >5 year old populations in Europe (figure 1.6) and is globally important as an invasive serotype.

Table 1.1 Pneumococcal capsular polysaccharides contained in the new generation of pneumococcal vaccines.
The serotypes are colour coded to show overlap between the vaccines. The heptavalent vaccine serotypes are included in all of the vaccines. The extra serotypes for the 9-valent vaccine are shown in red, for the 11-valent in blue and the 13-valent in green. Serotype 6A is underlined since it only appears in the 13-valent vaccine.

vaccine	serotypes covered	protein carrier
23-valent, polysaccharide vaccine (Pneumovax™)	1, 2, 3, **4**, 5, **6B**, 7F, 8, 9N, **9V**, 10A, 11A, 12F, **14**, 15B, 17F, **18C**, 19A, **19F**, 20, 22F, **23F**, 33F	N/A
Heptavalent – conjugate vaccine (Prevenar™)	4, 6B, 9V, 14, 18C, 19F, 23F	CRM_{197} OMPC
Nonovalent	1, 4, 5, 6B, 9V, 14, 18C, 19F, 23F	CRM_{197}
Eleven valent	1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	CRM_{197}
Thirteen valent	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F	?

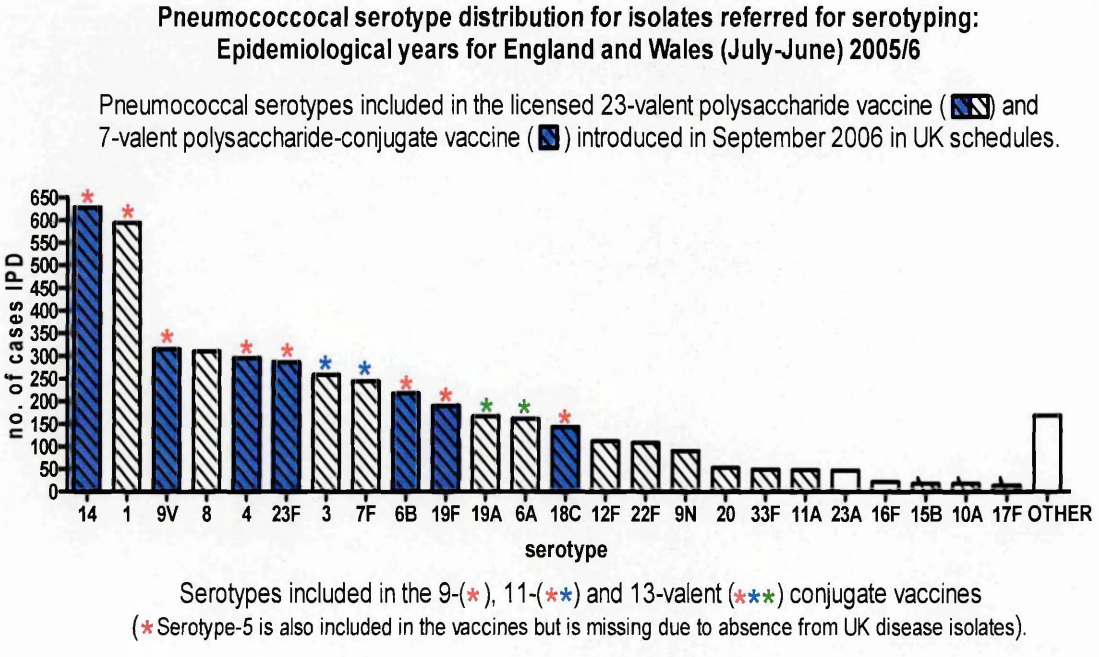


Figure 1.8 Coverage of IPD serotypes in the UK by current and new generation pneumococcal vaccines.
The data show the 24 most commonly isolated serotypes during 2005-2006, prior to the introduction of the Pnc7 vaccine into the infant immunisation schedule. Serotypes covered by the 23-valent polysaccharide vaccine (clear/blue hatched columns), the heptavalent (blue hatched columns only), 11-valent (red asterix) and 13-valent (green and red asterix) conjugate vaccines are indicated with the exception of serotype 5 which was not isolated during this period.

1.4.1 Pneumococcal polysaccharide vaccines

The capsular polysaccharides contained in this vaccine were based on those most responsible for invasive disease plus some cross-reacting serotypes (88). A number of serotypes such as 12F, 8, 22F, 15A, 15B and 11A are responsible for invasive disease and are included in the 23PsV but not in the newer conjugated vaccines (table 1.1)(69). The potential coverage of the 23PsV was found to be within the region of 90% of all IPD isolates in industrialised nations (89).

However the efficacy of the vaccine has not been so impressive with only 60%-70% responders in healthy, young adults and even lower in the >65 year olds (30%-80% efficacy against IPD) depending on underlying conditions (89, 90). The antibody induced by 23-PsV in the >65 years olds decreased to negligible levels for all but the most immunogenic serotypes by ten years post immunisation (91).

Finally the polysaccharide vaccine was ineffective in infants under 2 years of age (92-97).

Thus the populations most at risk from IPD (figure 1.5b) were not effectively protected by the polysaccharide vaccine.

A number of studies have looked in more detail at the response to the 23-PsV vaccine, concentrating on the antibody isotype (98, 99), subclass(100-102), function (103-105) and antibody secreting cells (ASC) (106-114).

Many of these studies were performed to establish the effects of age and/or underlying physiological condition on the immune response to the 23-Ps vaccine.

It was observed that increasing age was detrimental to the function of the antibody generated when compared to healthy young adults (105, 115, 116). The proportion of IgG2 induced by plain polysaccharide vaccines also increased with age (100, 101, 117).

Administration of 23-Ps to individuals with underlying conditions such as infection with the human immunodeficiency virus (HIV)(118), asplenia (119, 120), nephritic syndrome, asthmatic

bronchitis (121) and non-Hodgkins lymphoma (NHL) (122), resulted in altered immunogenicity of individual serotypes within the 23-Ps vaccine. The results from these studies generally showed reduced antibody longevity and function compared to age matched controls.

1.4.1.i Thymus independent (TI) response

The polysaccharide capsules of the 90, pneumococcal serotypes are conventionally called T cell independent type-2 (TI-2) antigens. TI-2 antigens have multiple repeating epitopes (glycotopes in the case of polysaccharides), that are capable of stimulating B cells in the absence of cognate T cell help.

The TI-2 responsive B cell subsets include marginal zone B cells (MZB), IgM-memory B cells and B1a and B1b cells and follicular (FO) B cells, all of which are discussed in later sections. These cells provide rapid innate and adaptive responses (123) to initial polysaccharide encounter and produce large amounts of IgM antibody some of which shows evidence of somatic hypermutation in the absence of T cell help (124, 125). The antibody response is generally short-lived and boosting fails to elicit increased antibody concentrations.

The TI-2 B cell responses occur in the extrafollicular regions of the lymph node medullary cords or splenic bridging channels (126). They form foci of proliferating antigen specific B cell clones that differentiate into short-lived, low avidity antibody secreting plasma cells (126-128). The lack of direct antigen specific T cell help in these areas means that there are no appropriate co-stimulatory signals to induce class-switching, somatic hypermutation of immunoglobulin (Ig) variable (V)-genes, memory B cell or long-lived plasma cell formation (129, 130). This process occurs within the first 3-4 days following immunisation or infection.

Some studies have questioned the lack of a role for T cells in polysaccharide responses (131-134).

For example, glycotopes are able to associate with the MHC-class-1 like molecule, CD1c. CD1c is expressed at high levels by MZB enabling them to present polysaccharide epitopes to γδ-T cells that may elicit a helper response (124, 135).

Zwitterionic polysaccharides (with negative and positively charged moieties) are able to associate with MHC class II (135). Examples of such polysaccharides are the capsules from pneumococcal serotype 1 and 3 (136, 137). Ordinarily polysaccharides inhibit lysosomal processing and do not bind the cleft of MHC class II (138). However, pneumococci with zwitterionic polysaccharide capsules are taken up by APC and killed via the nitric oxide (NO) pathway (136). These polysaccharides are then processed prior to normally required endosomal-lysosomal fusion as a neutral rather than acidic pH is required (136). The processed polysaccharide is then able to localise in the binding cleft of MHC class II (HLA-DR) and be presented at the cell surface (136, 137).

Pneumococcal polysaccharides have been shown to associate with dendritic cells (DC) and are taken up in endosomal compartments leading to altered cytokine production that may ultimately affect the outcome of the antibody response (139).

Finally, another marginal zone B cell surface receptor, SIGN-R1 (specific intracellular adhesion molecule-grabbing non-integrin-R1), binds the capsular polysaccharide of serotype-14 with high affinity, leading to uptake of the polysaccharide and activation of the MZB (140).

In children <2 years of age, the poor immunogenicity and longevity of the antibody response to 23-Ps immunisation has been suggested to result from an immature splenic marginal zone and possibly an immature bone marrow stromal cell environment (141-143). The marginal zone of the spleen is probably the major physiological site for anti-polysaccharide responses generated against encapsulated, blood borne bacteria, (144) and is the site of germinal centre (GC) formation in older children and adults (145, 146).

The lack of efficacy of 23PsV in the elderly and children <2 years of age has led to the generation of pneumococcal glycoconjugate vaccines with the aim of enhancing the immune response to IPD causing serotypes.

1.4.2 Conjugate vaccines

Since children <2 years of age make poor TI-2 immune responses but effective T cell dependent (TD) responses to protein antigens, the immune response to polysaccharides has been successfully manipulated by joining the polysaccharide to a carrier protein to create glycoconjugate vaccines (147). Common carrier proteins are tetanus toxoid (tet), diphtheria toxoid (dip), mutant diphtheria toxoid-cross-reactive material (CRM_{197}) or outer membrane protein complex (OMPC) of *Neisseria meningitidis* (148, 149).

The original bacterial conjugate vaccine was introduced to protect infants and young children against invasive disease caused by *Haemophilus influenzae* Type B (HiB) (148, 150). The vaccine targets the capsular polysaccharide polyribosyl ribitol phosphate (PRP) and contains between 3-10 repeating units joined to a carrier protein to enhance its immunogenicity (148). Inclusion of this vaccine into the childhood immunisation programme led a to dramatic fall in disease rates and in carriage, that in turn led to herd immunity in the unvaccinated population (151). On the basis of this success new pneumococcal conjugate vaccines (Pnc) were developed, containing 7-, 9-, 11 or 13 capsular polysaccharides, each individually conjugated to a carrier protein (Table 1.i).

The Pnc7 has led to reduced rates of carriage and invasive disease in infants since its introduction into the USA immunisation schedules (152-157). Figure 1.9 shows how dramatically rates of IPD dropped following the Pnc7 introduction in the Northern California Kaiser Permanente

population, for all children <5 years old (152, 158). Also of note is the slight increase in rates of non-vaccine and cross-reactive serotype IPD during the same vaccination period (152, 157). Pnc7-induced herd immunity has also reduced transmission of the bacteria from infants to other age groups resulting in a decline in disease rates in adults (159, 160). A study in the United States revealed a 93% reduction in invasive disease as well as reduced rates of pneumonia and otitis media (161).

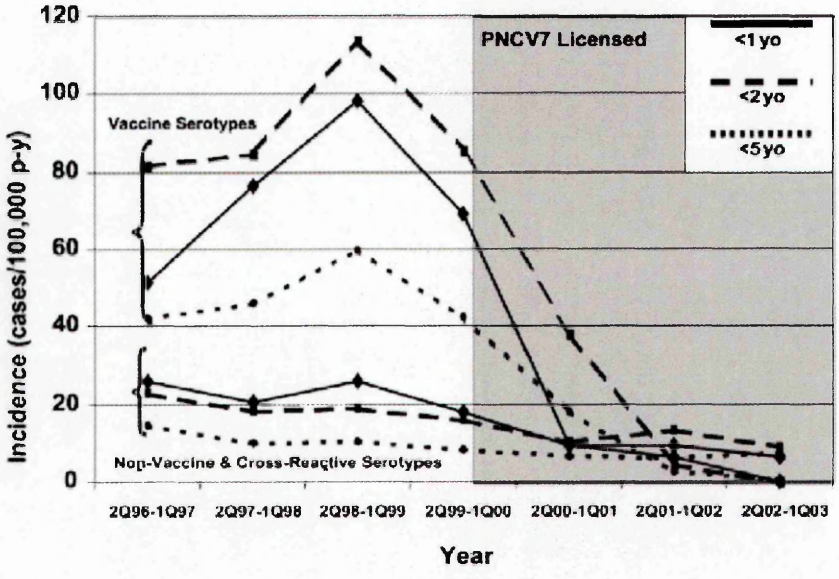


Figure 1.9 The effect of Pnc7 on rates of IPD in the <5 year olds in Northern California
(Black *et al*, figure 1 (152)). The chart shows the annual incidence of IPD for April 1996-March 2003. (2Q=2nd quarter-1Q=1st quarter).

In comparison to the original 23PsV, Pnc vaccines are highly immunogenic in infants and toddlers <2 years of age (studies 2, 4, 5, 6, 7, 8 and 9 (152, 157, 162-166), Table 1.2). The Pnc vaccines are also immunogenic in older children and adults with underlying conditions such as recurrent infections (study 3 (167, 168)), or with HIV (169, 170), Table 1.2). Pnc7 vaccine successfully primes infants and toddlers to mount strong amnestic responses to subsequent booster immunisation with 23-Ps at 12-18 months (studies 2 and 7 (165, 166), Table 1.2), but only against Pnc7 serotypes and not unrelated serotypes that are included in 23-PsV booster doses

(171). A similar effect has yet to be demonstrated in adults >50 years of age where immunisation with a Pnc5 vaccine failed to prime adults for stronger booster responses to 23-PsV (study 1(172), Table 1.2).

One major issue with the Pnc vaccine is that co-administration of other routine childhood immunisations appears to have a detrimental effect on the immune response to the polysaccharide antigens, particularly when Pnc containing tetanus toxoid as the carrier protein and diphtheria-tetanus-acellular Pertussis (DTaP) is given at the same time (studies 4 and 9 (157, 163), Table 1.2).

Three UK studies showed that the benefit of introducing the Pnc7 vaccine into the UK immunisation schedule would cut IPD rates by >60% (64, 72, 173). However, coverage of disease causing serotypes by the Pnc7 vaccine would be 79-84% in infants and <66% in adults >65years old (64, 72).

The current trends for pneumococcal disease for the UK and the coverage that might be obtained with the introduction of the heptavalent conjugate vaccine are shown in figure 1.10 (black line for the serotypes covered by the vaccine). It can also be seen from this data that an equivalent number of cases have occurred due to non heptavalent vaccine serotypes (red line), and it remains to be seen whether full vaccine coverage will have the same success in the UK as was seen in the USA.

The Pnc7 vaccine was introduced into the UK vaccine schedule in September 2006 (indicated by the green markers in figure 1.10). Two doses are to be given to infants at 2 and 4 months of age with a 3rd dose at 12 months of age. A catch up campaign was also carried out using a single dose at 12 months of age. The original USA schedule was for 4 doses (2, 4, 6 and 12 months), but due to shortages only 31% of infants received the full primary schedule, 74% received 3 doses (152).

Studies on number of doses have suggested that reduced dose schedules in infants and toddlers would suffice: i) a single dose at an optimal age of 5-7 months could protect against 15-62% IPD in the USA (174) and ii) a reduced, 2 dose schedule for priming was comparable to 3 dose priming in infants and a single dose in toddlers (175).

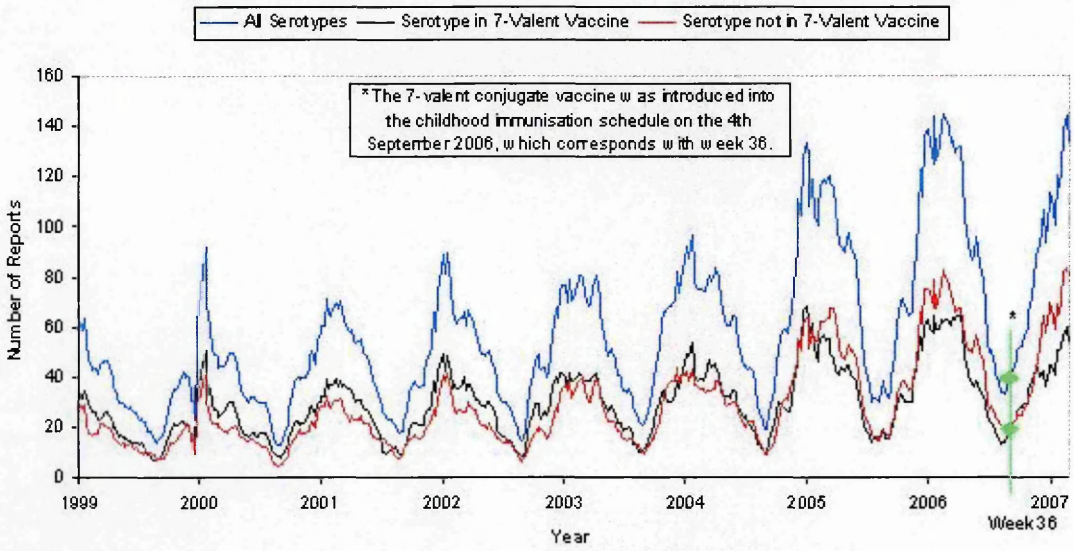


Figure 1.10 Seasonal fluctuation in pneumococcal disease in the UK reported to the HPA between 1999-29-03-2007.
(http://www.hpa.org.uk/infections/topics_az/pneumococcal/IPD5WMA.htm)

Poor coverage of some serotypes in Western Europe and developing countries prompted development of nine- and eleven-valent conjugates (table 1.i)(176) and they improved serotype coverage dramatically (72).

Recent observations indicate the need for vaccines which provide broader protection. For example, there has been an increase in carriage of non-vaccine serotypes and also invasive disease caused by these serotypes since the introduction of the heptavalent vaccine (177). Increasing frequency of non-vaccine serotype carriage may be due to an expansion of asymptomatically carried pneumococcal serotypes, or be the result of genetic transformation via horizontal transfer of capsule genes, a process known as capsule switching which can be

identified by MLST (81). Thus, benefits of immunisation with these multivalent vaccines maybe diluted by the emergence of new serotypes not previously seen as major causes of disease.

Table 1.2 Studies demonstrating the effectiveness of pneumococcal conjugate vaccines (Pnc) in all ages versus polysaccharide vaccines (23PsV) - on two pages).
Vaccines - DTaP (diphtheria-tetanus-acellular Pertussis) or DTwP (whole cell Pertussis), IPV (inactivated polio vaccine), HbOC (Haemophilus B conjugate).

Study	Vaccine	Study	Age group	Immunogenicity/efficacy	ref
1	23-valent polysaccharide vaccine (23PsV), (see table 1.1). Pnc5-CRM197 (Pnc5) 10μg -6B,14,18C,19F,23F	Comparison of IgG following - Pnc5 followed 6 months later by 23PsV boost or, - 23PsV followed by saline boost	≥50 years old	Responders (IgG ≥2 fold increase) were equivalent in both groups except against serotype 19F (Pnc5 was lower than 23PsV p=<0.05). Booster dose 23PsV only moderate increases in IgG, no different to single dose 23PsV. Pnc5 did not prime >50 year responses to 23PsV	Powers-1996(172)
2	Experimental conjugates 2μg/10μg doses of 23F,6A+CRM197 (native, Ps or oligosaccharide, OS). 23PsV booster	Priming with conjugate vaccine vs 23PsV. 23PsV booster 11-20 months post primary course	18-30 months of age	Conjugate priming induced 13-40 fold increases in GMC IgG following 23PsV booster. 23PsV priming (2-4 fold increases). 23F response > 6B response (primary and secondary) Primary response IgG1 Booster response IgG1+IgG2	O'Brien-1996 (166)
3	Pnc7	a) 23PsV responders vs 23PsV non-responders re-immunised with single dose Pnc7, or b) primed with 2 doses of Pnc7 vs 23PsV both with 23PsV booster	a) 12-13 years old with recurrent respiratory infections b) 2-18 year olds with impaired TI-2 responses	a) Responders to 23PsV GMCs = GMCs of non-responders who received Pnc7 re-immunisation Pnc7 immunogenic in 23PsV non-responders but not quite a good as in 23PsV responders b) Pnc7 primed for booster responses to 23PsV even in original non-responders to 23PsV.	a) Sorensen-1998 (167) b) Rose-2005 (178)
4	Pnc7	Immunogenicity of Pnc7 Effect of concurrent administration of DTaP/IPV/HbOC	2, 4, 6 and 12-13 months	Post primary IgG levels: >90% had >0.15μg/ml and 51%(type 9V)-89%(type 14) >1μg/ml Post booster IgG levels – increased for all serotypes Concurrent administration of HbOC and DTaP reduced booster responses to Pnc7.	Shinefield-1999 (157) Rennels-1998 (179)
5	Pnc7	Direct and indirect effects of Pnc7 on IPD in vaccinated children <5yrs of age and unvaccinated older children and adults	All children <5yrs of age 5->60yrs of age	Dramatic reductions in IPD in the <5yr olds (figure 1.9) caused by vaccine types. In >5yr age group by 2002-2003 there was a 25% reduction in all IPD with the largest reduction in 20-39 year olds	Black-2004 (152)

Study	Vaccine	Study	Age group	Immunogenicity/efficacy	ref
6	Pnc-9-CRM_{197} (Pnc9) 2µg-1,4,5,9V,14,18C,19F,23F +4µg 6B – CRM_{197}	Effect of Pnc9 on new carriage acquisition	12-17 months – 2 doses, 2mnth apart 18-35months – 1 dose	Serotypes 9V, 14, 19F, 23F Higher IgG correlated with lower acquisition (p=0.006 and 0.024 for 14 and 19F)	Dagan-2005 (162)
7	Pnc9 and 23PsV	Priming with Pnc9 or placebo followed by booster with Pnc9 vs 23PsV	6, 10, 14 weeks and 18 month booster	Pnc9 priming induced significant IgG responses to all serotypes, remained higher than controls at 18 months Pnc9 booster in primed group: IgG GMC 1, 6B,14,19F,23F rose significantly 23PsV booster-induced higher IgG responses in Pnc9 primed children then unprimed	Huebner-2004 (165)
8	Pnc -11 (DT/TT) 1µg -1,4,5,7F,9V,19F,23F-TT 3µg -3, 14, 18C+10µg 6B - DT	a) Comparison in Finish and Israeli infants b) In Filipino infants	a) 2,4,6 and 12 months Blood 1 month post primary and post booster b) 6, 10 and 14 weeks of age	a) Post primary: Highest GMC 4>7F>19F Lowest GMC 6B>18C Post booster: >80% infants >1µg/ml except for serotype 6B. b) At 18wks 100% infants = >1µg/ml IgG to all but serotypes 6B, 14, 18C and 23F (49, 83, 91.5, 87.2% respectively).	a) Dagan-2004 (164) b) Capeding - 2003 (180)
9	Pnc-11 (DT/TT)	Co-administration with DTaP/IPV/PRP-T or DTwP/IPV/PRP-T	2,4,6 and 12 months Blood 1 month post primary and post booster	aP reduced immunogenicity of Pnc-11-TT conjugates post 1 and boost (all serotypes) Pnc -11-DT (18C and 14)post boost wP – adjuvant activity masked these effects	Dagan-2004 (163)
10	Pnc-11 (DT/TT) vs 23-Ps	Pnc -11 with and without aluminium hydroxide adjuvant	age 26 (22-35) years	IgG – 6B, 14 and 19F in both Pncs Pnc higher IgG than 23-Ps except for types 1+9V Pnc +adjuvant IgG better for Ps-DT Pnc -non adjuvant IgG better for Ps-TT IgG response mainly IgG2 for all vaccines except for 6B IgG1 in Pnc +adjuvant	Wuorimaa-2001 (181)

1.4.2.i Thymus dependent (TD) response and the role of cytokines in Ig class switching

The carrier proteins of conjugated polysaccharide vaccines recruit $CD4^+$ T cell help into the vaccine response. In the TD response $CD4^+$ T helper (Th) cells provide signals for the differentiation of naïve B cells into plasma cells and memory B cells along with the induction of class switching and somatic hypermutation (132, 159, 160, 182-186).

In the multi-valent pneumococcal conjugate vaccines, each capsular polysaccharide is independently conjugated to the carrier protein CRM_{197}, and this causes variability in immunogenicity and in the isotypes of antibody elicited (93, 157, 165, 187-189).

Following the intra-muscular administration of a glycoconjugate vaccine the individual polysaccharide-protein conjugates either free or bound by local tissue dendritic cells are transported to the local draining lymph nodes. Here the polysaccharide and carrier protein may be bound directly by antigen specific B cells via the B cell receptor leading to direct activation or they are taken up and processed by DCs and B cells, both professional antigen presenting cells (APC), and the protein carrier presented to local T cells in the context of MHC class II.

Naïve T cells are primed only by DCs expressing processed carrier protein peptides. Primed T cells are able to interact with activated B cells presenting carrier protein peptides on MHC class II. Thus previous exposure to carrier proteins, such as tetanus and diphtheria toxoid, enhances immunogenicity of conjugate vaccines and can boost carrier specific responses in previously immunised individuals (190).

However the cytokines produced by the activated T cells within the GC can determine the isotype and therefore biological function of the antibody induced following immunisation (figure 1.11).

Following BCR ligation by specific antigen, two further signals are required for initiation of class switch recombination (CSR). One is the interaction of CD40L (on the T cell) with CD40 (on the B cell). The second is that provided by cytokines that induce signals for B cell proliferation, differentiation and CSR to particular isotypes (191-193).

Following glycoconjugate immunisation the desired outcome is the generation of high avidity IgG1(102) although there is generally a mixed ratio of IgG1:IgG2 that is dependent on age and vaccine formulation (101, 102). Plain capsular polysaccharide and conjugate vaccines induce IgA2 production from polysaccharide specific B cells (98, 99, 194). Previous studies have shown differences in Ig isotype induction and immunogenicity of the individual polysaccharide-protein conjugates (188, 189, 195). In a recent murine study serotypes-19F and 14 were conjugated with CRM and the antibody isotypes induced were identified. The 19F-CRM conjugate induced high anti-CRM responses and an IgG1 mediated anti-19F response. The serotype 14-CRM conjugate elicited a poorer anti-CRM response and an equal proportion of IgG1 and IgG2a in response to the type-14 capsular polysaccharide (189). In a similar study, but in humans, the anti-CRM T cell response was equally robust for all of the CRM-polysaccharides combinations, while the anti-polysaccharide antibody responses were variable (188). Therefore it was suggested that the cytokine environment within the germinal centre, induced by the individual polysaccharide protein conjugates may differ depending on the polysaccharide conjugated to the protein (188).

One of the mechanisms contributing to this was suggested by McCool *et al* (93) where polysaccharides could alter the peptide specificity of the T cell response as a result of altered antigen processing pathways for individual serotype polysaccharides. This was also confirmed by Leonard *et al* (196) who showed that unconjugated CRM_{197} or CRM_{197}-serotype 14 or 18C were processed and presented more efficiently than the other vaccine type conjugates. Finally, in a study from 2004 it was shown that serotype 14 polysaccharide conjugated to PspA protein induced $CD4^+$ T cell αβ TCR specific responses to both the protein and polysaccharide components of the conjugate (132).

Blocking of the signals between B and T cells either by monoclonal antibodies or by using gene knock out (k/o, -/-) mice have proven that CD40-CD40L interactions are essential for TD antibody responses (182, 193, 197-203). The main outcome of these experiments in murine models was a block in naïve B cell differentiation into plasma cells and immunoglobulin synthesis (201, 204). More specifically there was no production of IgG1 or IgG2a in mice (203). Natural Killer (NK) cells are also capable of providing the CD40-CD40L interaction to B cells, mainly to the innate $CD5^+$ B cells leading to IgM production (198).

Therefore in the presence of the appropriate co-stimulatory signals cytokines secreted by T, B and NK cells direct the antibody response. In mice and humans there exist different $CD4^+$ T cell subsets that are identified by the cytokines they secrete. Figure 1.11 shows how the differentiation of these $CD4^+$ T cell subsets is induced. Production of IL-12 and IL-27 by APC induces T helper type 1 (Th1) $CD4^+$ T cell differentiation while the secretion of interleukin (IL)-4 by APC in the germinal centre environment can induce a T helper type 2 (Th2) response in the absence of interferon-gamma (IFNγ) (205). In humans the subsets of $CD4^+$ T cells includes Th1, Th2, Th17 and T regulatory (Treg) cells, figure 1.11 (206). Th1 cells secrete IFNγ, tumour necrosis factor (TNF), IL-2 and granulocyte macrophage-colony stimulating factor (GM-CSF). Th1 cytokines in humans induce antibody isotypes IgG1 and IgG3 while in mice IgG2a and IgG2b result. Th2 cytokines induce IgG2 and IgG4 in humans and IgG1 in mice (191, 203). Figure 1.11 shows how the Th1 and Th2 responses interact to regulate one another so that IFNγ suppresses Th2 responses and IL-4 suppresses Th1 responses (191, 206, 207).

IL-10, produced by $CD4^+$ Treg, is a major Ig-gene CSR factor (192, 193, 199, 208). In conjunction with CD40-CD40L co-stimulation the targeted naïve B cell differentiation into memory B cells is blocked by IL-10 with preferential differentiation into plasma cells(209, 210) that secrete IgM, IgA and IgG1, 2, 3 and 4 (192, 193, 208). However, IL-10 in the presence of

IL-4 increased CSR from IgM to IgG but there was no secretion of antibody, while IL-4 alone induced IgE CSR and secretion (192, 193). IL-10 with IL-13 resulted in no Ig CSR while IL-13 with IL-4 resulted in IgE and IgG (192, 193). Therefore it appears that IL-10 negatively regulates Th2 TD responses (211). TGFβ alone or in combination with IL-10 results in IgA1 and IgA2 production (192, 193).

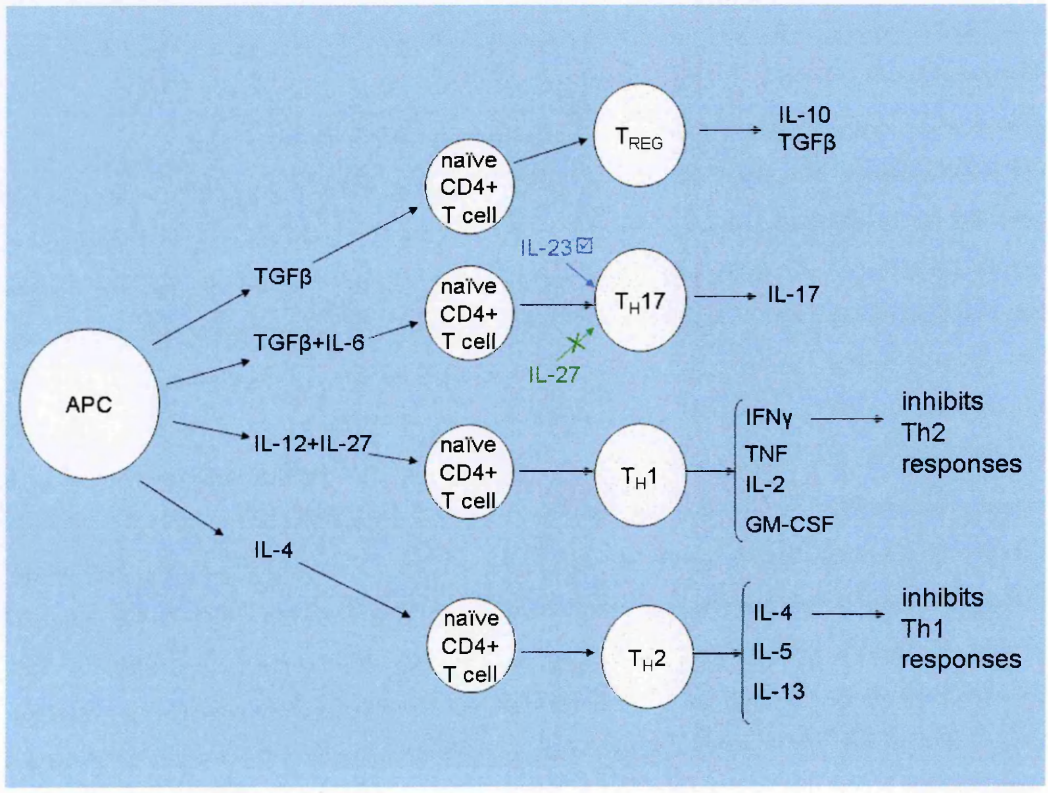
Th1 cytokines IL-2 (212, 213) and IFNγ enhance IgG synthesis and B cell growth and differentiation.

Another regulatory cytokine, IL-21, has recently been implicated in B cell differentiation (200, 214-216). It is produced by peripheral blood $CD4^+$ T cells (200, 216) and NKT cells (216) along with follicular $CD57^+CXCR5^+$ T cells (200, 217). The receptor for IL-21 (IL-21R) is expressed highly on naïve and to a lesser extent on memory B cells (214) and is also found on MZB (215), some T cells and NK cells (200, 216). The effect of this cytokine is to enhance proliferation, differentiation and CSR in B cells, particularly naïve B cells, but again requires B cell co-stimulation via CD40-CD40L (200, 214). Th1 cytokines (IL-2) enhance the activity of IL-21 while Th2 cytokine (IL-4) inhibit it (200) and in combination with B-lymphocyte stimulation protein (BAFF), IL-21 induces MZB differentiation into plasma cells (215).

Finally a subset of $CD45RO^+$ T cells that secrete IL-17 (called Th17 cells) have an unknown role in immunoglobulin synthesis but are known to have a proinflamatory role inducing IL-6, IL-8, TNFα and GM-CSF production (218). IL-23 is essential for IL-17 secretion (219). This entire process can be blocked by the presence of IL-27 that in combination with IL-12 is a potent inducer of Th1 cell differentiation (220). Therefore it seems that IL-27 promotes beneficial production of Th1 cytokine responses to specific antigens such as tetanus toxoid and purified protein derivative, while IL-23 promotes a more pathogenic inflammatory process mediated IL-17 mediated production of inflammatory responses in organs and neuronal tissues (in the absence of IFNγ) without promoting Th1 responses (218, 220). Thus in figure 1.11 the balance of cytokine production is described with Th1 and Th2 pathways being important for antibody class

switching and regulated by Treg produced IL-10. Further discussion of cytokine involvement in vaccine responses can be found in chapter 3.

T cells have also been shown to recognise carbohydrate antigens. T cell clones that were specific for the carrier protein (tetanus toxoid) and also to the meningococcal group C polysaccharide (MCPS) were generated in response to immunisation with a meningococcal group C-tetanus toxoid conjugate in mice. While the TT clones were found to be conventional MHC class II restricted $CD4^{+}\alpha\beta$ TCR clones the MCPS clones were also $CD4^{+}\alpha\beta$ TCR phenotype but unrestricted by MHC class II although requiring direct contact with APC for *in vitro* proliferation (221).



1.11 Factors that help determine the differentiation of naïve, CD4+ T cells into four major subsets (Treg, Th17, Th1 and Th2 cells) (206).

1.5 Host immune response to initial pneumococcal exposure

1.5.1 Recognition of colonising bacteria

Interaction between the pneumococcus and the host occurs initially in the nasopharyngeal mucosa during transient carriage. Colonising bacteria at the mucosal surface are detected via pathogen recognition receptors (PRRs) that bind highly conserved bacterial components, or pathogen associated molecular patterns (PAMPS) (15, 222). A family of PRRs called the Toll Like Receptor (TLR) family have a role in the innate immune response to pneumococci.

TLR-2, 4, and 9 have been identified as having synergistic roles in immunity to IPD (11, 223).

TLR-2 is the receptor for lipoteichoic acid and peptidoglycan and mediates the immune response to pneumococci by enhancing the response in synergy with other TLRs (223). Challenge of TLR-2 knock-out mice resulted in increased bacterial cell numbers in the brain (18) when compared to control mice.

TLR-4 is involved in inflammation induced by pneumolysin (45, 224, 225) and absence of TLR-4 increases susceptibility to NP colonisation.

TLR-9 binds un-methylated cytosine-phosphate-guanosine (CpG) DNA sequences that are released during bacterial cell lysis and induce B cell activation (226, 227). TLR-9 expression is localised to the endosomal compartment of epithelial cells and APC such as dendritic cells and B cells but expression is only up-regulated by naïve B cells following antigen receptor cross-linking (228, 229) and then remains expressed by memory B cells. Therefore phagocytosis and lysis of bacteria allows CpG-DNA to bind TLR-9, enhancing the activation signals to the immune cells (230). A particular synthetic CpG sequence called CpG2006 (CpG motifs in bold) (5' T**CG**-T**CG**-TTT-TGT-**CG**T-TTT-GT**C**-**G**TT 3') has been identified as ideal for activation of human B cells *in vitro* (229, 231, 232).

1.5.2 Mucosal IgA response to pneumococcal carriage

Pneumococcal colonisation of the nasopharynx generates a predominantly IgA mediated response (233). IgA binds to pneumococci or the breakdown products of lysed bacterial cells and these are then targeted for phagocytosis via the FcαR1 on macrophages or the antigen-antibody complexes are transported to the mucosal surface via the pIgR transcytosis mechanism (41). Therefore IgA appears to be a critical evolutionary first line of defence against colonisation (234) and pneumococcal protein-specific IgA can be detected in saliva of children as young as 6 months of age, possibly induced by carriage (61). IgA can be induced in a T cell independent manner, via secretion of transforming growth factor-beta (TGF-β) from non-lymphoid cells which is able to induce class-switch recombination in $IgM^{+}IgD^{+}$ B cells toward IgA expression (235).

1.5.3 Clearance from the mucosa

Until recently it had been assumed that anti-capsular antibody mediated clearance of pneumococci from the nasopharynx. This inference was based on the correlation of serum antibody levels increasing with age and frequency of carriage while IPD rates decreased. Also, pneumococcal vaccines induced elevated serum antibody concentrations that correlated with reduced carriage (37, 55, 236, 237).

However, it has recently been proposed that clearance of pneumococci from the nasopharynx involves mechanisms that are independent of capsule specific antibody. The proposed mechanism is a combination of innate immune responses, anti-surface protein antibody and $CD4^{+}$ Th1 cells (38, 49, 224, 238-241).

McCool *et al* demonstrated that in humans exposure to and subsequent carriage of an attenuated serotype 23F pneumococcal strain was unaffected by the hosts serum antibody level. However

they did find that NP exposure to the same strain induced anti-PspA IgG in the serum and secretary IgA at the mucosa. They also found that those who did not carry the serotype already had higher levels of pre-existing anti-PspA IgG and IgA than those who did develop carriage (238). In a later study this group also demonstrated that only the more variable surface proteins (PspA and CbpA) were accessible to the host immune system and that these proteins induced antibody that correlated with protection from carriage (239).

1.5.4 Immunity to blood borne pneumococci

Once the pneumococcus enters the blood stream the PCh residues in the CwPS bind to CRP (222) which, in turn, activates the classical complement pathway leading to opsonisation (47). Complement bound to CwPs is able to cross-link the B cell Receptor (BCR)-co-receptor complex and complement receptors (CD21 and CD35) on B cells, leading to enhanced activation signals and natural antibody production by the specific B cell (242, 243). The anti-CwPs and anti-PCh antibody can be IgM or IgG and accumulate with age (6, 244) induced by the repeated exposure to pneumococcal serotypes through NP carriage (244).

Natural IgM antibody binding the bacterial cell surface induces classical complement activation and deposition of C3b and blocking of this mechanism in mice leads to rapid septicaemia (245). Therefore, anti-CwPs-Ab provides a first line of defence against blood borne IPD, though the mechanisms involved in this protection are unclear since the ability of these antibodies to opsonise and target the bacterium for phagocytosis is poor (16). One possibility is that antibodies directed against CwPS neutralise the inflammatory effects induced by the breakdown of this molecule (6) thereby decreasing pathogenicity.

Another PRR-PAMP innate mechanism for clearance of blood borne pathogens involves mannose-binding lectin (MBL). MBL fulfils the role of capsule specific Ab, opsonising the polysaccharide capsule and activating complement, while the bacterium is in the spleen or liver, and induces phagocytosis. LPS binding protein (LBP) also has a role in protection of the host

against IPD since it binds to the peptidoglycan, a component of all bacteria providing broad, non-species specific immune recognition (11).

1.5.5 The role of dendritic cells

The innate immune system provides a regulatory pathway for the adaptive immune response to pneumococcal infection through the role of DC (246). DCs recognise non-self molecules (PAMPs) via the TLR mediated recognition of danger signals, such as heat shock proteins (hsp), bacterial DNA and surface antigens (247).

Invading pneumococci activate epithelial cells, NK cells and mast cells that are then induced to secrete hsp. DCs constantly survey the mucosal surfaces and become activated in response to these danger signals (246). They are phagocytic cells that engulf invading bacteria or bacterial cell breakdown products and then migrate to the local secondary lymphoid tissues. In the nasopharynx the local lymphoid tissues are the tonsils or adenoids, while for blood borne pathogens, such as pneumococci, the major lymphoid organ is the spleen (248).

It has been suggested that different DC subsets will determine the outcome of the T cell response (247). For example, DCs that home to the Peyers patches of the gut or those in the respiratory tract may help prime a Th2 response, while those in the spleen may skew the response of T cells toward a Th0/Th1. DCs interact with MZB in mice to induce TI-responses, which may also be the case in humans (248). DCs are also involved in the response of memory B cells. Myeloid DC express BAFF that allows the survival of peripheral naïve B cells and memory B cell derived plasma cell survival(249). This allows CD40-independent Ig class switching in response to TD and TI antigens.

1.6 B cell subsets mediating T cell independent responses to pneumococcal polysaccharides.

1.6.1 IgM memory

IgM memory is an important immunological mechanism that provides rapid natural and antigen specific memory responses. Initial protection from encapsulated bacteria such as S. *pneumoniae*, H. *influenzae* and N. *meningitidis* and other blood borne infections such as *Borrelia hermisii*, is mediated by IgM. IgM memory increases from birth, peaking during young adulthood and declining again in the extreme elderly (fig1.12) (144, 250-253).

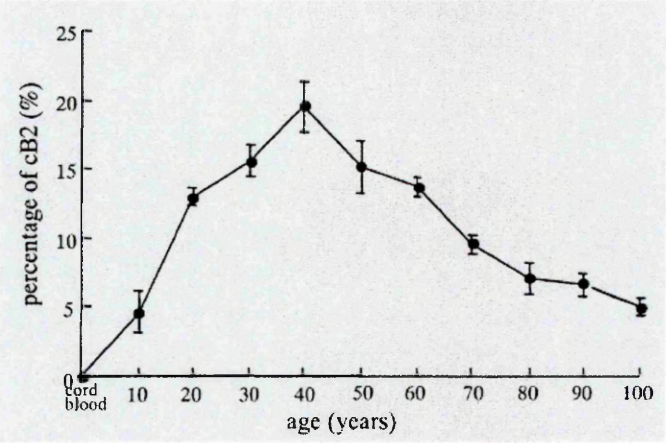


Figure 1.12 Frequency of $IgD^+IgM^+CD27^+$ B cells (CB2 or MZB) in human peripheral blood. PBMCs were isolated from 140 healthy donors aged (0-99years). From Y. Shi *et al*, figure 6(250).

Following primary exposure to carbohydrate antigens activated B cells differentiate into non-proliferating, short-lived plasma cells (PC) that secrete antibody (predominantly IgM), with germline gene arrangements, by day 5 of the immune response (145, 254-258) and disappear from the peripheral blood by two weeks after vaccination (259-262). These cells re-circulate through the peripheral blood, reside mainly in the spleen or peritoneum and have a half-life of 3-5 days (263). This early phase of the humoral response occurs in the absence of germinal centre formation and without direct interaction with T cells. Therefore there is no generation of classical

memory B cells, although there may be some class switching of secreted antibody to low avidity IgG1 or IgA (256-258, 263-266). Furthermore, repeat immunisation of adult humans with plain polysaccharide vaccine does not result in enhanced immune responses or new generation of antibody forming cells (AFC) (108, 267). Pneumococcal polysaccharide-protein conjugate vaccines on the other hand induce B and T cell memory and long term antibody production by recruiting T cells in a TD response(93, 188, 189).

There are three main B cell lineages, the B1a cells, B1b cells and the B2 cells that interact to control the early stages of infection, providing long term immune memory to protect from re-infection (268-271). B1a and B1b cells mediate the innate immune system, while the B2 cells (marginal zone and follicular B cells) provide the adaptive immune response.

BCR signalling is required for survival of all B cells (272) and it is the strength of antigen receptor signalling that determines the pathway along which the B cell precursors develop. Strong signalling through the BCR generates B1 B cells, while signalling of intermediate strength leads to FO B cell development and weak signalling, involving the Notch2 pathway, induces MZB development (272, 273). Baker *et al* (274) suggested that the concentration and quality of the naturally occurring IgM can regulate the B cell subset that differentiates from immature B cell precursor in the spleen. In their murine studies, high concentrations of polyclonal, cross-reactive IgM results in a reduction in the numbers of B1 and MZ B cells and an expansion of FO B cells as a result of enhanced BCR signalling (123). Other surface molecules help to regulate differentiation of these subsets. Those that inhibit or impair BCR signalling select for MZB cells development, for example CD22 which inhibits antigen receptor signalling (275) and CD21 (complement receptor (CR)2)(276). An absence of CD22 allows the development of FO B cells (277, 278) while the absence of CD21 leads to MZB cell development (276). Likewise, CD19 enhances BCR signalling and absence leads to MZB development impairment (279).

1.6.2 B1 cells ($CD5^{+/-}$ B cells).

The B1 cells appear earliest in mammalian B cell development, arising from progenitor cells in the foetal liver. They are maintained as a self replenishing population in the peritoneal and plural cavities of mice and humans throughout adulthood (280). Studies in asplenic mice revealed that the spleen may also be essential for survival and self-replenishment of B1 cells in mice (281). In mice, the expression of $CD45^{lo}$ ($B220^{lo}$) IgM^{hi} $CD23^{-}CD43^{+}IgD^{lo}$ (282) distinguishes the B1 cells from conventional B2 cells. The B1 cell population is then further subdivided in mice and humans, based on the surface expression of the CD5, into B1a ($CD5^{+}$) and B1b ($CD5^{-}$) cells (283, 284).

The developmental lineage of B1 cells was clarified more recently by Montecino-Rodriguez *et al* 2006 (270) who identified B1 cell progenitors in mice with the phenotype of $Lin^{-}CD45R^{lo-neg}CD19^{+}$ (B1 cell progenitor) while the conventional B2 cell progenitor is $Lin^{+}CD45R^{+}CD19^{-}$. They found the B1 progenitors were present at high frequencies in murine foetal liver but at very low frequency in adult bone marrow (figure 1.13). They were also able to show that the B1 progenitor isolated from adult bone marrow only differentiated into B1b cells not B1a cells thus confirming three separate B cell lineages in mice (269-271).

The role played by the B1 cell subset as a whole is rapid generation of poly reactive IgM antibody (and also IgA and IgG3 (282)), that targets highly conserved, PAMPs, such as PCh residues found in CwPS of all pneumococcal serotypes, phosphatidyl choline (PtC), lipopolysaccharides (LPS) and influenza virus antigens (282, 285). B1 cells do not express terminal deoxynucleotide transferase (TdT), thus immunoglobulin heavy and light chain genes are expressed as germline sequences with no somatic hypermutation (SHM) (282). As well as recognising PAMPs, the antibody produced by B1 cells can also react with specific antigens such as the polysaccharide capsules of encapsulated bacteria like *Streptococcus pneumoniae* (282, 286, 287). B1a cells are

thus, responsible for the rapid and early production of natural antibody specific for PAMPs, the role of which is probably to mediate complement-antibody mediated opsonisation and localisation of capsular polysaccharide antigens to the marginal zone, allowing cross-linking of the BCR co-receptor (CD19/CD21) complex (288). The B1b cells then generate antibody in response to antigen challenge, providing a longer lasting response to specific antigens such as pneumococcal polysaccharides, influenza virus proteins and *Borellia hermisii* antigens (268, 289, 290). Further still, in a murine study, natural IgM antibody specific for influenza haemagglutinin was detected prior to infection (the B1a cell response) but did not increase following infection, while IgM produced by B2 cells rose by days 7-21 (290) suggesting that the innate and adaptive arms of the humoral response interacted to provide protection from influenza virus infection.

The expression of CD5 and CD19 surface molecules are thought to act independently to regulate the development of the B1a and B1b cell responses. CD19 knock out mice only develop B1b cells and are susceptible to infection in the absence of prior exposure of the mouse to the specific antigen (268). CD5 expression provides a mechanism for stimulation of B1a cells that is independent of BCR signalling, maintaining survival in the absence of proliferation of the B1a cells (291, 292) and this may be related to NFκB expression levels (293, 294). It was also suggested that CD5 is an activation marker rather than a lineage marker (291, 295) that targets B cells for apoptosis following BCR ligation (296). In a recent human study it was shown that CD5 was down regulated by Epstein Barr Virus (EBV) infected B1a cells preventing apoptosis of the transformed B cell (296). On the other hand, higher levels of CD5 expression have been noted on B cells of HIV^+ individuals (297).

Proliferation and differentiation of B1 cells into Ig-secreting cells is also maintained by IL-5 secreted by helper T cells and mast cells (285). In mice stimulation of B1 cells and IgD^+ splenic B cells by IL-5 induced the production of IgG1 (298). Importantly, since B1 cells mediate the innate humoral response to invasive bacterial and viral pathogens, TLRs are thought to regulate

the activation and migration of this B cell subset without the need for BCR ligation (299, 300), providing a mechanism for maintenance of natural IgM and mucosal IgA levels.

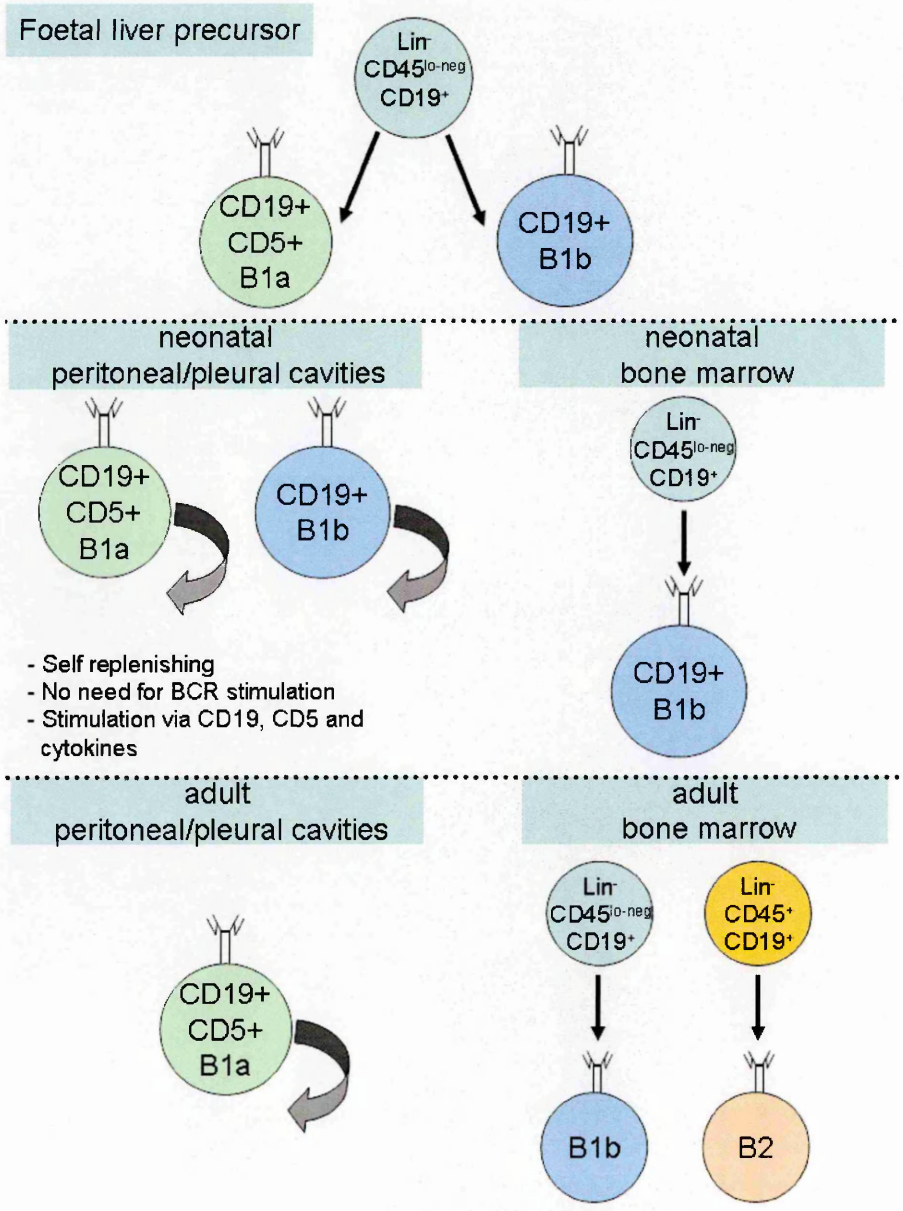


Figure 1.13 A scheme of B cell subset development from specific progenitor cells in mice, that may be similar in humans.

1.6.3 The B2 cells (marginal zone and follicular B cells).

MZB cells and follicular (FO) B cells are separate developmental B cell lineages that, in mice, appear to originate from the same precursor cell (274). The newly formed (NF) B cell precursor (also called T1), develops in the marginal sinus of the spleen and then migrates to the follicle under the influence of CXCL13 (the ligand for CXCR5/3). Here the NF B cell precursor differentiates into T2-FP precursor that, in turn, becomes a follicular B cell ($IgM^{hi}IgD^{hi}CD1d^{lo}CD21^{int}CD23^{+}$) or into a second, very similar precursor T2-MZB that becomes a MZB ($IgM^{hi}IgD^{lo}CD21^{hi}CD1d^{hi}CD23^{lo}$) (273, 301).

Thus, in the spleen and lymph nodes of mice there are two or three subsets of B cells with the $IgD^{hi}CD23^{hi}$ phenotype (FO, T2-FP and T2-MZB in the spleen and FO, T2-FP in the lymph nodes). It is possible that this may also be the case in humans were the MZB cell phenotype is $CD27^{hi}IgM^{hi}IgD^{lo}CD21^{hi}CD23^{-}CD1c^{hi}$. In mice and in humans the MZB and FO B cells can be identified by the level of surface IgD expression where FO B cells are IgD^{hi} and MZB are IgD^{lo} (252, 302, 303). MZ and FO B cells are also distinguished by the expression of surface CD9 and Fc receptor homolog (FcRH)-3(304).

1.6.3.i Marginal zone B cells

The MZB cells have recently been associated with responses to TI-2 antigens such as pneumococcal polysaccharides (123, 252). They contribute to the initial, rapid formation of plasmablasts in response to blood borne pathogens such as pneumococci, providing a first line of defence against blood borne pathogens (123, 305). MZB cells exist in a highly activated state compared to FO B cells, including higher levels of Blimp-1(306) that is required for rapid differentiation into plasmablasts (123). Human MZB cells are also distinguished by the high level expression of CD38, CD9, CD25, and migratory markers such as β2 integrin, LFA-1 and α4β1 integrin (301, 307). Expression of these markers further confirms that MZB cells are not a resting cell but are poised to mount rapid responses to invasive pathogens (301, 307).

MZB cells have been identified in rodents and humans but in rodents these cells are limited to the spleen and do not re-circulate (273), while in humans the MZB cells reside in and home to the marginal zone of the spleen, but re-circulate through peripheral blood and are found in other secondary lymphoid organs (252, 253).

The TI-2 response is dependent on the presence of a mature and functional spleen (308). It has previously been demonstrated in mice and humans that loss of the spleen, splenic hypofunction or congenital asplenia leads to a loss of the IgM^+ memory population (144, 309) resulting in higher rates of disease caused by encapsulated bacteria (281).

Therefore residence of MZB cells in the splenic marginal zone means that they are ideally situated to respond to blood borne pathogens. In humans, the marginal zone separates the outer red and inner white pulp of the spleen and has been subdivided into inner and outer areas based on the presence of specialised fibroblasts and helper T cells (146). Lymphocytes re-circulate through the spleen, via the central arterioles that open into the perifollicular zone, between the marginal zone and outer red pulp (figure 1.14).

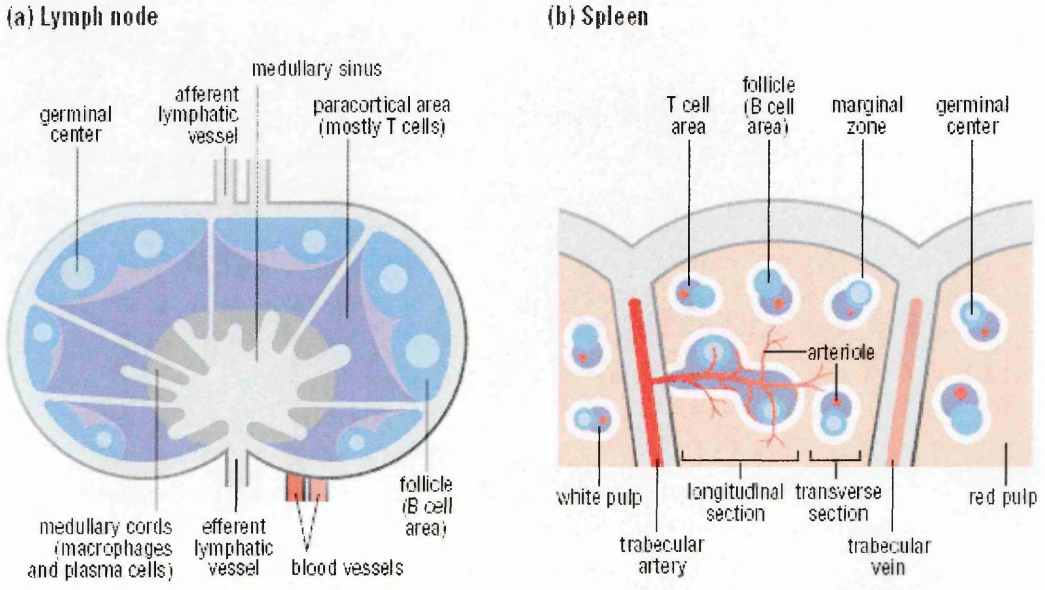


Figure 1.14 Structure of the secondary lymphoid tissues of the lymph node and spleen
http://www.sinauer.com/pdf/nsp-immunity-1-6.pdf
Chapter 1, 2007 New York Science Press Ltd, Overview of the immune response, page 15

Reticular fibres arise from the central arterioles, forming bridging channels between the red and white pulp. Specialised marginal DC reside there while **blood derived DC (CD11c^{lo})** and neutrophils that bind whole bacteria or soluble bacterial polysaccharides (among other antigens) on their surface migrate to the bridging channels of the spleen where they present the polysaccharide antigen to the MZB cells (248). It has recently been demonstrated that the capsular polysaccharides are preferentially sequestered in the marginal zone of the spleen (139). Surface lectins on DCs bind the carbohydrate antigens and enhanced migratory capacity allows these cells to re-locate to the spleen. Exposure of these particular DCs to pneumococcal antigens has been shown to induce up-regulation of B lymphocyte stimulator (BlyS or B cell activation factor, BAFF) and APRIL (A proliferation-inducing ligand), that interact with BAFF-R (receptor) and TACI (transmembrane activator and calcium modulator cyclophilin interactor) and BCMA (B cell maturation factor) on the B cell. BLyS and APRIL are soluble factors that bind to BAFF-R, TACI or BCMA and this is required for plasmablast formation (310-314). The interaction of MZB cells with DCs induces rapid proliferation and differentiation of the MZB into antibody secreting plasmablasts.

In response to viral infection in mice it was shown that the MZB (CD23^{hi}CD21^{hi}) rapidly responded with class switched IgG specific for the virus by day 5 after challenge while the follicular B (CD23^{lo}CD21^{lo}) cells did not exceed the MZB contribution until three weeks after challenge (307, 315). This process has also been shown in mice where the MZB provide rapid ASC formation that switches from IgM to IgG by day 8 post challenge while the FO B cells rapidly form germinal centres but no ASC appear until day 8 after challenge (123, 301).

Marginal zone macrophages (in rodents) also play an important role in retaining antigen specific lymphocytes in the marginal zone. They present polysaccharide antigens via surface receptors such as sialic-acid binding lectins (Siglecs), presenting conformational epitopes to the B cells. However, this mechanism has not been demonstrated in humans (146).

MZB cells increase in frequency with age (figure 1.12), but have an immature phenotype in infants under the age of two years. The functional difference between MZB in the under 2 year olds and adults involves the lack of CD27 and CD21 expression in the marginal zone (144, 316). Before the age of 2 years children have an immature splenic marginal zone structure that is unable to support $CD27^+$ MZB cells, (the lack of CD27 expression in the marginal zone of infant spleens has been demonstrated in several studies (316, 317)), and renders infants incapable of mounting rapid secondary responses (316). CD21 (complement receptor 2) is highly expressed on mature MZB cells but not on MZB from children <2 years of age. CD21 binds the C3d component of complement that opsonises bacterial capsular polysaccharides and failure to engage CD21 at the same time as BCR ligation reduces the activation signal received by IgD^+IgM^+ B cells and may induce apoptosis of MZB cells binding polysaccharide via the BCR(316, 318).
The combination of low MZB frequency in this age group with reduced MZB responsiveness may be among the reasons for elevated rates of IPD caused by blood borne pathogens (317).

CD27 and also CD148 are markers for human memory B cells (319) and approximately 40% of human peripheral blood B cells are memory ($CD27^+$) B cells and within this memory population 50% express class-switched (IgG, IgA, IgE), immunoglobulins. The remaining 50% of peripheral blood memory B cells consist mainly of $IgM^+IgD^+CD27^+$ B cells with a small number of IgM only or IgD only memory cells (146, 252, 273, 320, 321). On FO B cells CD27 indicates previous germinal centre development and it is required for Ig class switching and somatic hypermutation. However, as already described, CD27 is expressed by mature MZB cells in the adult splenic marginal zone (146, 322). The peripheral blood MZB cells resemble IgM^+ memory cell phenotype ($IgM^+IgD^+CD27^+$), and express somatically mutated surface IgM (320).
The immunoglobulin produced by MZB cells undergoes CSR more rapidly (by day 5 after antigen challenge (307)), than is seen in FO B cell switched (IgG^+) and IgM-only memory

populations, (3 weeks post challenge, (307, 323)), and there is also enhanced switching to IgA production in mice (324).

Although CSR is more rapid in MZB, SHM occurs more slowly and in a smaller percentage of cells than is observed in FO B cells following immunisation (325). Willenbrock *et al* (325) and Weller *et al* (321) were able to demonstrate that activation induced cytidine deaminase (AID), that is required for SHM to occur was expressed at levels 20 times less than was seen in switched memory FO B cells.

MZB cells ($IgD^+IgM^+CD27^+$ memory cells) with somatically mutated Ig are present in patients with X-linked hyper-IgM syndrome, where cognate T cell interactions required for B cell differentiation, germinal centre formation and isotype switching are absent (125, 252). The lack of T cell help is the result of CD40L deficiency, preventing signalling between the B and T cell which drives class switch recombination and somatic hypermutation. The presence of MZB which are still able to mutate their immunoglobulin receptors, suggests an alternative pathway exists for TI CSR (252, 267, 324). Weller also found that although infant MZB cells were expanded and mutated, they were still incapable of making TI responses.

As well as responding to TI-antigens MZB cells respond to TD-antigens as well. Protein antigens are processed and presented in the context of MHC class II (expressed at high levels on MZB). Following antigen up take and presentation by MZB cells the expression of costimulatory molecules CD80/86 (B7.1/2) is rapidly upregulated, turning them into potent APC for $CD4^+$ T cells. Attanavanich *et al* (326) showed that this skewed the T cell response towards a Th1 cytokine secretion pattern in mice. Despite the interaction with T cells the response of MZB to TD antigens remained extrafollicular, occurring in the absence of germinal centre formation, at the T cell zone-red pulp interface. There is some CSR and SHM but not to the same extent seen following a germinal centre reaction (123, 315).

1.6.3.ii The follicular B cell response.

Following immunisation with either a glycoconjugate or plain polysaccharide vaccine tissue resident, immature DC encounter antigen as immune complexes (ic), capture it via complement and FcR and become activated, adopting a mature phenotype (327, 328). These mature DC migrate to the secondary lymphoid organs such as spleen and lymph node (figure 1.14) (327, 328). These DC are able to recycle antigen to and away from the cell surface so that it can be presented to B and T cells and is retained for long periods of time (130, 329). Expression of high affinity BCR on the surface preferentially selects a B cell for extrafollicular differentiation into plasma cells while lower affinity BCR selects the B cell for GC development (127, 330). Extrafollicular differentiation of B cells into plasma cells provides early antibody that can bind antigen, creating more ic for capture by follicular dendritic cells (FDC) (331, 332).

Germinal centre formation occurs in the B cell areas of secondary lymphoid organs (333), and they are comprised of a dark zone where antigen specific B cells rapidly proliferate and a light zone (basal and apical) where centrocytes undergo SHM and CSR (334).

Germinal centre precursor B cells migrate to the B cell follicles under the influence of CXCL13, expressed by reticuloendothelial cells in the B cell follicles (217, 335, 336), where they localise at the border of the T cells zone (328, 337). Here the GC precursors become **follicular mantle zone (FM) B cells** ($IgD^{+}CD38^{-}CD40^{+}CD23^{+/-}$) that are activated following interaction of the surface immunoglobulin (sIg) with specific ic bound to interdigitating DC (iDC) (130). The naïve follicular mantle zone B cells become $IgD^{+}CD38^{+}IgM^{+/-}$ GC founder cells that undergo intense proliferation but very little immunoglobulin variable (V)-gene mutation(130, 329). The proliferating focus of B cells becomes the GC dark zone and the GC founder cells lose IgD expression and become $CD77^{+}$ centroblasts that continue to proliferate intensively. Thus GC formation is antigen dependent beginning with the clonal expansion of just a small number of B cells and also T cell dependent (130).

The interaction of specific antigen with sIg leads to up-regulation of CD80/86 on the B cell surface that provides the initial co-stimulatory contact with CD28 on primed GC T cells brought in to close proximity with the B cells via the iDC. Re-circulating, antigen specific T cells become trapped either by MHC class II plus peptides on iDC (for naïve T cell priming), or on newly activated B cells.

The B cell-T cell interaction inturn, leads to the up-regulation of CD40 ligand (CD40L/CD154) on the T cell surface and this interacts with CD40 on the B cell. CD40-CD40L interaction is essential for B cell selection and death, proliferation, differentiation of B cells, SHM and CSR of immunoglobulin genes (130, 329). The proliferating centroblasts also up-regulate surface CD27 expression(130, 329). The interaction of B and T cells via CD40-CD40L occurs at 2-3 days following antigen encounter, such as glycoconjugate immunisation(202, 338) and is the signal for the GC reaction to begin by day 4. The expression of CD40L is transient and disappears within 72 hours, thereby providing the initial stimulus for B cell clonal expansion and germinal centre formation (129). CD27 is essential for continued germinal centre reactivity, memory B cell and plasma cell differentiation (under the influence of IL-10) and antibody secretion (182, 183, 339). It enhances the interaction with GC T cells and is found in both the germinal centre and splenic marginal zone. $CD27^+$ B cells have higher rates of somatic mutation and class switching, especially to IgG (340). This period of intense proliferation in the GC dark zone and up-regulation of co-stimulatory molecules ensures that a large number of progeny are generated that then undergo the selection processes based on the affinity of the mutated Ig for the antigen(130, 329).

Naïve B cells receiving stimulation via CD40-CD40L interaction predominantly differentiate into memory B cells with some plasma cell formation. If naïve B cells do not receive BCR stimulation and CD40-CD40L interaction then they are likely to undergo apoptosis, particularly after the FM B cell stage when Bcl-2 (a survival factor), is lost and apoptosis factors such as Fas,

Bax, p53 and c-myc are up-regulated. Centroblasts express particularly high levels of c-myc (331, 332, 341).

Some GC B cells undergo a permanent switch from **constant(c)μ to cδ** (IgD^+IgM^-) phenotype and express high levels of Fas/CD95 following which they undergo antigen affinity based selection based on the BCR affinity for the antigen. High affinity interactions leads to clonal expansion and differentiation and no or very low affinity induced apoptosis (130, 329).

Shortly after this selection stage the ability to undergo SHM with each cell division is acquired by the centroblasts (130, 329). During this phase the Ig V-gene sequences acquire random mutations so that the expressed sIg has an altered sequence from the original clones (130, 329). The IgD^+ IgM^- $CD38^+$ B cells have much higher rates of V-gene mutations than seen in the later gamma gene sequences (130, 329).

Following affinity maturation of the antigen receptor and down regulation of c-myc and the up-regulation of p53 the cells leave the cell cycle to become $CD77^-$ sIg^+ centrocytes (130, 329, 331, 332, 341-343).

The centrocytes progress to the basal light zone, expressing fully mutated antigen receptors and associate with a sparse population of T cells (333). The centrocytes are then selected on the basis of the affinity of the sIg for specific antigen captured on the surfaces of FDC and also molecules expressed on the surfaces of GC T cells (327). Here the B cells then localise with FDC ($CD23^{hi}$) that form a network for the capture of free ic from the lymph through out the apical light zone that is processed and presented on the FDC surface. High affinity receptor centrocytes are also positively selected by capturing free ic via their sIg. (127, 130, 329, 330) that is processed and presented to follicular T helper (TFh) cells ($CXCR5^+CD57^+$) that populate the apical light zone.

GC T cells ($CXCR5^+CD4^+CCR7^+$) initially reside at the T cell border but migrate to the B cell follicles in response to CXCL13, the ligand for CXCR5 (217, 335, 336). In the GC light zone the sparse TFH cell population localises with the centrocytes around FDC, providing CD40L

interactions, secretion of IL-4, IL-10 and IL-21, TGFβ, and FasL expression, all of which provide even more efficient help for centrocyte antibody synthesis (217, 335, 336) and differentiation into memory B cells or back to centroblasts in the dark zone (130, 329, 343). The sparsity of these TFH cells limits B cell-T cell interactions preferentially selecting high affinity B cell clones that express processed antigen on their surface (344).

There is still a high rate of apoptosis at this stage because centrocytes either; 1) do not meet their specific antigen, or 2) the mutated BCR is no longer avid enough, or is autoreactive, and undergo deletion due to Fas-Fas-ligand (FasL) interaction or low signal strength in the absence of T cell help as very few are present at this location (130, 329, 343). The centrocytes apoptose and are removed by tingible body macrophages(130, 329, 343).

It is at this stage that class switching of the surface Ig occurs. Thus, B cells with unsuccessful Ig-V-gene mutations are sensitive to apoptosis and only B cells with high avidity sIg for the immunising antigen emerge from the germinal centre to differentiate into memory or plasma cells.

1.6.3.iii Products of the germinal centre

The GC reaction then lasts for approximately 3 weeks (appearing by day 4 and involuting by day 21-28) relying on either the continued presence of antigen on FDC surfaces for its maintenance or on the decay of the B cell proliferation stimulus to limit the GC reaction (333, 334, 345, 346). The peak in GC activity occurs during the second week after antigen challenge (days 7-10) (333, 334, 346) following secondary immune responses or booster immunisations and results in the formation of 3 subsets of B cells: Long-lived plasma cells expressing CXCR4 that home to the bone marrow in response to CXCL12; Non-secreting plasma cell precursors that remain in the secondary lymphoid tissue; and memory B cells that express switched Ig molecules and are $CXCR5^{+}$ which allows homing to the B cell follicles so that they can re-circulate between blood and lymph (145, 217, 254, 263, 347, 348).

Generation of long lived plasma cells and memory B cells is the basis for maintenance of long term antibody production(142, 143, 145, 255-258, 264, 299, 338, 348-360). These studies have all demonstrated the importance of long term survival of plasma cells in the bone marrow for maintaining antibody levels. In infants and young toddlers the microenvironment of the spleen is not mature enough to maintain the plasma cell survival for long (142, 143, 350, 356). However once the bone marrow environment is able to support survival of these plasma cells it is still possible for them to be dislodged from their niches (257, 348). Down regulation of CXCR4 on bone marrow plasma cells means that they are unable to re-circulate and they eventually die in the periphery. Constant arrival of cells of new specificity without renewal of the old specificity cells may eventually lead to depletion of plasma cells of some specificities (257, 348).

Another mechanism for maintaining long term memory is the polyclonal activation of memory B cells by bystander T cell activation of through polyclonal stimulants such as PAMPs or bacterial DNA (228, 351, 359).

1.7 Quantification of the humoral response to pneumococcal vaccine

A rapid rise in specific Ab following booster immunisation with a polysaccharide vaccine at 12 months of age demonstrates that primary immunisations with glycoconjugate vaccines in the first few months of life generate memory responses (165, 361). Sustained levels of capsule specific, serum IgG Ab (>0.2 μg/ml) (362) may indicate persistence of protection (187). However little is known about maintenance of protection if serum IgG concentrations fall below this putative threshold (0.2mcg/ml). In very young infants, after primary immunisation, levels of capsule specific antibody may fall below the limits of detection. Low levels of antibody may be present

but undetectable by conventional assays. However memory B cells may be present and provide protection at earlier ages than when antibody is first detected (37).

The quality of the antibody produced is also important and antibody avidity for solid phase antigen is another method for assessing vaccine effectiveness, with a rise in avidity demonstrating generation of B cell and T cell memory via germinal centre development and somatic hypermutation (97, 150, 363-366). Opsonophagocytic activity of serum prior to and following immunisation is also another parameter that can be assessed as it mimics the natural clearance mechanism of pneumococci and shows good relationship to ELISA based IgG avidity measurements (103, 104, 120, 367-369).

More recently antigen specific B cell responses have been studied in humans and mice. However, there is very limited human data tracking these responses following immunisation in humans. Existing data includes studies of smallpox, tetanus, diphtheria and pneumococcal vaccines (111, 188, 370-373). Antigen specific, spontaneously secreting B cells appeared in the peripheral blood between days 5 and 8 after immunisation in all of these studies (106, 111, 261, 374, 375).

It is suggested that these spontaneously secreting, antigen specific AFC are probably derived from memory cells residing in secondary lymphoid tissues after primary immunisations (145). Booster immunisation induces activation and rapid proliferation of vaccine specific, memory B cells (260) with both extrafollicular and germinal centre responses. The extrafollicular response results in a rapid efflux of antigen specific AFC into the peripheral blood, peaking on days 6-7 after immunisation providing initial protection from infection while germinal centres develop (145, 256, 264).

In-vitro stimulation of isolated B cells has allowed the detection of low frequency, antigen specific AFC which are putative memory or precursor plasma cells that persist for some time after antigen exposure. These cells, present in the peripheral blood, are distinct from spontaneously secreting cells and require added stimulation via the BCR and co-stimulatory

molecules before Ig is secreted (228, 259, 354). In-vitro stimulation with *Staphylococcus aureus* Cowan 1 strain (SAC) which cross-links the BCR, and IL-2 provides the signals necessary for the expansion of these antigen specific memory B cell populations, facilitating their detection by ELISpot (183, 372, 376, 377).

There is little data available on the kinetics of plasma and memory B cell responses following primary immunisations or primary exposure to infectious agents, therefore this thesis examined the phenotype and kinetics of B cell populations appearing in peripheral blood in relation to the isotype and magnitude of the specific serum immunoglobulin response following primary and booster immunisation with Pnc7.

1.8 Aims

The primary aims of this thesis were;

1. Could polysaccharide specific memory B cells be isolated from the peripheral blood of toddlers prior to immunisation with Pnc7 vaccine? Would immunisation with a glycoconjugate vaccine induce detectable increases in the frequency of these B cells?
2. Are the kinetics of the appearance of polysaccharide specific memory B cells and plasma cells in adult peripheral blood in response to immunisation with Pnc7 vaccine similar to those reported in previous studies?
3. What are the phenotypes of spontaneously secreting plasma cells and memory B cells induced in adults by glycoconjugate vaccines? Does this alter with increasing age?
4. Does age at time of immunisation affect the frequency of plasma cells and memory B cells generated in response to the polysaccharides contained in the Pnc7 vaccine?

Chapter 2: Materials and Methods

2.1 Antibodies

2.1.1 The ELISpot assay

2.1.1.i Coating the ELISpot plates (refer to section 2.5.1-2.5.3):

The capture antibody for total immunoglobulin (Ig) control in the ELISpot assay was a polyvalent goat-anti-human Ig (Caltag, H17000, Buckingham, UK) used at 10μg/ml in phosphate buffered saline (378).

2.1.1.ii Antibodies for the detection of IgG, IgA or IgM antibody secreting cell spots (refer to section 2.5.4):

The detection antibodies were goat anti-human IgG, γ-chain specific -alkaline phosphatase conjugate (#401442), goat anti-human IgA, α-chain specific-alkaline phosphatase conjugate (#401132), goat anti-human IgM, μ-chain specific-alkaline phosphatase conjugate (#401902; Calbiochem-Novabiochem, Nottingham, UK), all diluted at 1:5000 for use in the ELISpot development.

2.1.2 Fluorescent activated cell sorting (FACS) (refer to section 2.8).

All of the monoclonal antibodies (MAb) were raised in mice against human lymphocyte surface antigens and were of the murine IgG1 or IgG2a isotype. The fluorescent labels used were Fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin chlorophyll protein (PerCP), BD-Cy-chrome™ (PE-Cy5). All antibodies for FACS were obtained from Becton Dickinson

(BD Biosciences Ltd), Oxford, UK, except for anti-IgA-FITC, which was obtained from Calbiochem-Novabiochem.

2.1.2.i Isotype controls for non-specific binding;

Antibody isotype controls used were Becton Dickinson (BD) SimulTest^{TM}-isotype control (anti-IgG1-FITC plus anti-IgG2a-PE), and anti-IgG1-PerCP.

2.1.2.ii Antibodies against the following lymphocyte markers were used:

FITC-labelled anti-CD3, anti-CD27, anti-IgA, anti-CD21, anti-CXCR4 and anti-CD62L.

PE-labelled anti-CD27, anti-CD38, anti-IgG, anti-IgD. PerCP-labelled anti-CD20, anti-CXCR5, anti-CCR7 and PE-Cy5 labelled anti-IgM.

2.1.3 Detection antibodies for the pneumococcal serotype ELISA (refer to section 2.10).

IgG, IgA and IgM serum antibodies specific pneumococcal serotypes 4, 14 and 23F were detected using the following enzyme conjugates from Biosource CA. USA. All of the MAb were raised in goat against human serum IgG, IgA and IgM and all were conjugate to alkaline phosphatase (AP).

Goat-anti-human-IgG-AP (AHI0305), Goat-anti-human-IgA-AP (AHI0105) and Goat-anti-human-IgM-AP (AHI0605). The IgG-AP conjugate was used at 1:2000 and the IgA-AP and IgM-AP conjugates were used at 1:1000 as predetermined by prior dilution experiments.

2.2 Buffers

2.2.1 Phosphate buffered saline x10 concentration (PBSx10) for the ELISpot and pneumococcal serotype ELISA.

Sodium Chloride (NaCl, 80g/litre); potassium dihydrogen orthophosphate (KH_2PO_4, 3.14g/litre); di-sodum hydrogen phosphate – heptahydrate (Na_2HPO_4-$7H_2O$, 20.6 g/litre); potassium chloride

(KCl, 1.6 g/litre). All the powders were dissolved in 800ml of sterile, pyrogen free water and then the volume made up to 1 litre. The pH was then adjusted to 7.2-7.4, using 6M sodium hydroxide (NaOH) or 10M hydrochloric acid (HCl) and then stored for up to 6 months at room temperature.

2.2.2 PBSx1 for coating of ELISpot and pneumococcal ELISA plates.

The PBSx10 was diluted 1:10 using sterile pyrogen free water to make the required volume for the coating of either ELISpot or ELISA plates. The pH was then adjusted to 7.2-7.4, using 6M NaOH or 10M HCl and then stored for up to 6 months at room temperature.

2.2.3 Wash buffer for the ELISpot assay: PBSx1+Tween20(T) 0.25% (PBS-T0.25).

The wash buffer was made from PBSx1 plus the required volume of Tween20 to give a final concentration of 0.25% Tween20. For example 100ml of PBSx10 mixed with 900ml of sterile, pyrogen free water plus 2.5ml of Tween20. The pH was then adjusted using 6M NaOH or 10M HCl to 7.2-7.4 and the buffer was stored for up to 1 month at room temperature.

2.2.4 Pneumococcal serotype ELISA wash buffer: Tris-phosphate buffered saline with 1% Brij-35 solution (TBS-Brij35x10).

Sodium chloride (NaCl, 160g/2 litres); potassium chloride (KCl, 3.20g/2 litres); Trizma hydrogen chloride (Tris-HCl, 29.12g/2 litres); Trizma base (Tris-Base, 1.88g/2 litres) ; Brij-35 solution (1%, 66mls in 2 litres). All of the salts were dissolved in 1934ml of sterile pyrogen free distilled water. To this was added 66mls of the Brij-35 detergent solution, making up the final volume of

2 litres. The pH was then adjusted using 6M NaOH or 10M HCl to 7.2-7.4 and the buffer was stored for up to 1 month at room temperature.

2.2.5 Substrate buffers and stop solutions

2.2.5.i Substrate buffer for the ELISPot assay

Alkaline phosphatase substrate kit – 2 components (Biorad Laboratories 170-6432) made as per manufacturers instructions. The reaction was stopped with 200µl per well of sterile, pyrogen free H_2O.

2.2.5.ii Substrate buffer for the pneumococcal serotype ELISA

1M diethanolamine with 0.5 mM $MgCl_2$ (Don Whitley Scientific E00016) used with Phosphatase substrate 5mg tablets (4-Nitrophenyl phosphate disodium salt hexahydrate, Sigma S0942 tablets). The reaction was stopped with 50µl 3M NaOH per well.

2.2.6 RPMI for peripheral blood mononuclear cell (PBMC) isolation.

The RPMI medium was made using a 500ml bottle of RPMI-1640 medium with phenol red indicator, 25mM hepes modification with sodium bicarbonate (RPMI, Sigma R5886). This was supplemented with 5ml of L-glutamine at 2mM (Sigma G7513), and 5ml of penicillin and streptomycin (Sigma P4458) at 50U/ml and 0.05g/ml respectively. The medium was stored at 4°C until use, when it warmed to room temperature before use.

2.2.7 RPMI+newborn bovine serum (NBBS) at 10% (10%NBBS).

The 10%NBBS was made by supplementing the RPMI described above with 50ml of heat inactivated NBBS (Sigma N4637). This 10%NBBS was used for all cell culture medium and for

the ELISpot plate blocking step and cell incubations. The 10%NBBS was stored at 4°C until use when it was warmed to room temperature.

2.2.8 RPMI+human AB serum (HABS) at 10% (10%HABS).

The 10% HABS was made to the required volume by diluting the HABS 1:10 in RPMI under sterile conditions. This medium was stored at 4°C until use when it was warmed to room temperature.

2.2.9 PBS with 2mM Ethylenediamine tetraacetic acid (PBS-EDTA): Rinse buffer for the AutoMACS™ procedures.

Five PBS tablets (Sigma P4417) were dissolved in 1 litre distilled H_2O and then EDTA 2mM (0.744g EDTA/litre of PBS) was added. Once dissolved, the pH was corrected to 7.2-7.4 using 6M NaOH or 10M HCl and then was autoclaved. The rinse buffer was stored at room temperature for up to 6 months.

2.2.10 PBS-EDTA+0.5%NBBS: Cell wash buffer for cultured PBMC harvesting and also for the AutoMACS™ procedures.

A 1 litre bottle of sterile PBS-EDTA was opened in the laminar flow, cell culture hood and a 5ml aliquot of NBBS was added to a final concentration of 0.5%NBBS. The wash buffer was stored at 4°C and kept sterile for use.

2.3 Subjects and clinical procedures

2.3.1 Vaccines

A heptavalent pneumococcal-CRM_{197} conjugate vaccine (Pnc7, Wyeth Vaccines, Pearl River, MA), given by intra-muscular injection in the left deltoid. The 0.5ml dose of the vaccine contained polysaccharide concentrations of 2.0μg/ml of serotypes-4, 9V, 4, 18C, 19F, 23F and

4μg/ml of 6B. Each oligosaccharide is conjugated to CRM_{197} (mutant diphtheria toxoid) and adsorbed on aluminium phosphate.

Purified pneumococcal polysaccharide vaccine (23PnV, Pneumovax™, Merck) containing 25μg of each of the capsular polysaccharides for serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F.

2.3.2 Blood draw volumes

In the adult studies, venous blood samples of 20mls were split as follows, 18mls were collected in sterile, preservative free heparin tubes at each time point and 2mls were collected as a clotted sample, from which serum was obtained and stored at -80°C.

For the toddler studies, 5ml of venous blood was collected at each time point, 4mls into heparin and 1 ml for serum.

Informed consent was obtained from the volunteers and the protocol was approved by the Oxfordshire's Research Ethics Committee (OxREC number C02.005 (toddlers and young adults and OVG2006/01 (elderly adults)).

2.3.3 Age groups

The age of individuals enrolled into the studies conducted for this thesis fell into three groups. There were toddlers aged 12 to 14 months of age, young adults aged (32 (23-49) and elderly adults with a mean age of 58 (47-66) years old.

Details of the study groups can be found in the subjects and clinical procedures sections for each of the results chapters.

2.4 Density gradient separation of PBMCs from whole blood

PBMCs were separated from whole blood using the density gradient medium Lymphoprep™ (Axis-Shield). Fresh, whole blood was diluted 1:2 for 18ml blood volumes and 1:3 for the small infant blood sample volumes of 1-4ml using RPMI. The diluted blood was then layered over half its volume of Lymphoprep™. The layered blood was then centrifuged, with the break off, for 30 minutes at 2200rpm (1100xg). Following centrifugation the buffy coat was carefully removed to a clean tube and then washed again in RPMI for 20 minutes at 1800rpm (700xg). Following this wash step the cell pellet was re-suspended in the appropriate culture medium for counting or was washed a further three times in PBS+EDTA+0.5%NBBS for use in the *ex vivo* ELISpot, before counting.

2.4.1 Cell counts

Prior to use in the cell culture assay or ELISpots or FACS assays, the PBMCs were counted on a haemocytometer. For counting and exclusion of dead cells 50µl of cell suspension were diluted with 50µl of PBS and 50µl of trypan blue (0.4%). Following gentle mixing 10µl was removed and added to the haemocytometer. The number of cells per ml of suspension was then calculated as follows. Cells within 5 squares of the central haemocytometer grid were counted and then multiplied by 5 to give the number of cells in 25 squares. This was then multiplied by 3 to account for the (trypan blue+PBS+cells) dilution. This figure was then multiplied by 10^4 to give the number of cells/ml of PBMC suspension. The number of cells/ml was then divided by the required concentration of cells for the assay (e.g. $2x10^6$ cells/ml), to give the total volume of the cells at $2x10^6$cell/ml. The initial volume of the PBMC suspension was subtracted from this total volume to give the volume of 10%NBBS to be added.

2.5 The ELISpot assay for the detection of antigen specific IgG, IgA and IgM-antibody forming cells (AFC).

2.5.1 Control antigens

The assay positive controls were anti-Ig polyvalent antibody (section 2.1.1) for detection of the total IgG, IgA or IgM AFC and tetanus toxoid (tet) (606lf/ml or 1515μg/ml stock, Statens Seruminstitute, Copenhagen, DK) as a non-vaccine related antigen control (a negative control for the *ex vivo* assay and a positive control for the memory B cell assay). PBS alone was used as the background control for non-specific binding to the membranes and any spots detected were subtracted from the antigen specific well spot counts.

An unstimulated control was not included in the final assay based on previous evaluation. Spontaneous formation of IgG-AFC was evaluated during prior development of the ELISpot assay. PBMCs from ten unimmunised donors were cultured for five days to compare unstimulated (10%-NBBS) with stimulated (SAC+CpG+PWM) cells for IgG-AFC formation. The result of the unstimulated cultures was some spontaneous IgG-AFC formation in the total IgG control wells of the ELISPot with a median spot count 0 (0-500) IgG-AFC/million cultured PBMCs. None were detected in wells coated with serotype 4, 14 or 23F tetanus or diphtheria toxoid. Therefore the cut off for total IgG was set at ≥1000 spots per million IgG-AFC and for the specific antigens a positive response was >1 spot per million IgG-AFC.

2.5.2 Vaccine of vaccine related antigens

The vaccine related antigens used were diphtheria toxoid (dip) (2005lf/ml or 5012μg/ml stock), (Statens Seruminstitute, Copenhagen, DK), to show the CRM197 carrier response and pneumococcal purified polysaccharides from serotypes-4, 14, 23F (LGC Promochem, Teddington, UK), conjugated to mHSA (National Instititute for Standards and Controls, Potters

Bar, UK). Since availability of PBMCs was limited only 3 of the seven pneumococcal vaccine serotypes were studied.

2.5.3 Antigen coating concentrations

The ELISpot plates used in this assay were 96-well Multiscreen^{TM} plates with hydrophobic polyvinylidene fluoride (PVDF), membranes at the base of the wells (Millipore, Watford, UK). The polysaccharides were mixed with equal concentrations and volumes of mHSA as in the comix method described by Nelidia Concepcion *et al* 1998(379). All of the antigens were coated at 10μg/ml in PBS except tet and serotype-23F which were coated at 5 and 20μg/ml respectively. The plates were then sealed and incubated overnight at 4°C and then stored as such for up to one month.

2.5.4 The ELISpot assay

Before use, the plates were washed three times with 200μl/well of 1xPBS. The wells were then blocked with 200μl of 10%-NBBS for at least 30 minutes at 37°C in 5% CO_2 and 95% humidity. PBMCs were prepared as in section 2.4 to give a final suspension of $2x10^6$cells/ml. The cells were then seeded at $2x10^5$cells/well in then antigen specific wells and at $2x10^4$, $2x10^3$ and $2x10^2$cells/well for the anti-Ig control wells. The cells were then incubated for 16-20 hours at 37°C in 5% CO^2 and 95% humidity. Following the cell incubation the wells were washed four times with 200μl/well PBS-T0.25% and then once with 200μl/well 1xPBS. The bound antibody secreted by the cells was then detected by incubating the plates with one or all of anti-IgG, IgA or IgM alkaline phosphatase conjugates (all diluted at 1:5000), at 50μl/well, for 4 hours at room temperature. The wells were then washed four times with 200μl/well PBS-T0.25% and then three times with 200μl/well water. The bound conjugate was then revealed using 50μl/well of the AP-conjugate substrate kit (nitroblue tetrazolium + 5-Bromo-4-chloro-3-indolyl phosphate in dimethylformamide, Bio-Rad Laboratories, Hemel Hempstead, UK), and stopped using

200µl/well of water. The plates were then dried and scanned for counting using an AID ELISpot reader ELR02 and software version 3.2.3. (Cadama Medical Ltd, Stourbridge, UK). The parameters for accepting spots were preset following optimisation of the spot counts prior to counting all plates in the studies. The spot characteristics were set to include thresholds of the following parameters: Spot intensity (minimum of 10), spot size (minimum of 40), spot emphasis (tiny). The well saturation level was set to a maximum of 70% before the well was uncountable. Debris was edited out of the wells manually to avoid false positive readings. Any spots detected in the PBS control wells were subtracted from the total-IgG control and the antigen specific wells. Samples were only accepted when the total-IgG control spot count was $\geq$1000 spots/10^6 PBMCs.

2.6 *In vitro,* polyclonal stimulation of PBMCs for the expansion of memory B-cells

PBMCs were isolated from peripheral blood using density-gradient centrifugation as described in section 2.4 and re-suspended at 2x10^6 cells/ml in 10%-NBBS as described in section 2.4.1.

The polyclonal stimulants used were combinations of Staphylococcus aureus cowan 1 strain plus IL-2 (SAC+IL-2) or SAC plus CpG-2006 plus pokeweed mitogen (PWM) (SAC+CpG+PWM). The final well concentrations of the stimulants were as follows; SAC at 1:5000 dilution of the Pansorbin™ commercial cell suspension (Calbiochem-Novabiochem, Nottingham, UK), IL-2 at 50U/ml (Roche Diagnostics stock concentration 10,000U/ml, Mannhiem), CpG-2006-ODN at 2.5µg/ml (Autogen Bioclear, UK) and PWM at 83ng/ml (Sigma L-9379, UK). The stimulants were diluted in 10%-NBBS and 100µl volumes were added to an appropriate number of wells in a round bottom, 96-well culture plate. Following this the PBMCs were added in 100µl volumes giving a final cell density of 2x10^5cells/well. The PBMCs were then stimulated, *in vitro* for 5 days at 37°C in 5% CO_2 and 95% humidity.

2.7 Preparation of *in vitro* stimulated PBMCs for IgG, IgA and IgM ELISpot assays.

Following 5 days of stimulation the PBMCs were harvested by gentle re-suspension and transfer to a clean tube where the contents of the wells for each individual sample were pooled. The cells were washed once in PBS+EDTA+0.5%NBBS at 2000 rpm (900xg) for 20 minutes to remove cell culture supernatant. The cells were then washed three times in PBS+EDTA+0.5%NBBS at 1800rpm (700xg) for 15 minutes to remove residual antibody secreted during the 5 day stimulation culture. Following this the cells were resuspended in 1ml of 10%-NBBS and counted as in section 2.4.1. The cells were then resuspended to $2x10^6$ cells/ml and were then put in to the ELISpot assay described in section 2.5.

2.8 Flow Cytometric phenotyping of PBMCs

PBMCs separated as in section 2.4, were assessed for expression of surface markers CD3, CD4, CD16, CD19, CD27, CD56, CD62L, CCR7, IgD, IgM and IgG (see section 2.1.2 for details). A standard procedure was used for labelling the cells. Briefly, 1x105 PBMCs were incubated with pre-determined volumes of the above antibodies in three colour combinations that would allow analysis of memory B-cell subsets as well as general lymphocyte populations. The cells were labelled for 15 minutes in the dark, at room temperature. The cells were then incubated in BD FacsLyse™ for 5 minutes and then washed at 1500rpm (475xg) for 5 minutes, followed by a wash in BD CellWash™ (PBS+EDTA), at 1500rpm (475xg) for 5 minutes. The cells were finally fixed in 200µl of BD CellFix™ (1% formaldehyde).

The labelled cells were then run on a BD FacSCAN™ (three colour flow cytometer), that was able to detect green, red-orange and far-red fluorescence. The data was collected and analysed using BD CellQuest™ software version 3.1. The number of events collected was 10,000 unless

the sample was too small and then the count was stopped manually at 3-5000 events. A gate was set on the lymphocytes and the subsets within the lymphocyte gate were subsequently analysed.

2.9 Separation of PBMCs for the detection of Ig-secreting cells by ELISpot.

PBMC subsets were positively selected using an AutoMACs™ cell separator (Miltenyi Biotec Ltd. Bisley, UK), on the basis of CD20, CD27, CD38, IgA, IgD, CXCR4 or CXCR5 expression using the microbeads as per manufacturer's instructions (Miltenyi Biotex Ltd, Bisley, UK). Briefly the PBMCs (between $1x10^6$ and $1x10^7$ cells depending on the sample size), were incubated with the antibody coated magnetic beads for 15 minutes at 4°C. The bead-cell mixture was then washed once with PBS+EDTA+0.5%NBBS at 1500rpm (475xg) for 10 minutes. The bead-cell pellet was then resuspended in 500µl of PBS+EDTA+0.5%NBBS in preparation for passing through the AutoMACS™ column. The programme used was a positive selection, double column separation (Posselds) preset by Miltenyi Biotec. This programme involved passing of the positively selected sample through a second separation column for extra purity of the cell fraction. Both the negative and positive fractions were counted and resuspended to known concentrations that varied due to the size of the pre-exisiting subset in the whole PBMC fraction. The separated fractions where then seeded on to antigen coated ELISpot wells as described in section 2.5. The results were expressed as the percentage spots/well to take into account to differing cell fraction sizes. The un-separated PBMC fraction was always run along side the separated fractions as a control.

2.10 An ELISA for the detection of pneumcoccal polysaccharide capsule specific immunoglobulins in human serum.

The World Health Organization standard protocol was used for detection of human IgG, IgA and IgM antibodies against *S. pneumoniae* capsular polysaccharides (380), http://www.vaccine.uab.edu). In brief, serum or plasma samples were pre-absorbed with 10μg/ml of CPS (Statens Seruminstitut, Copenhagen, DK) and 10μg/ml of a non-vaccine related serotype polysaccharide, 22F (LGC Promochem, Teddington, UK). The standard serum, 89SF (supplied by the Federal Drug Administration) was used to create the standard curve for the ELISAs and control sera were obtained from NIBSC, Potters Bar, UK. Table 2.1 shows the predetermined IgG, IgA and IgM levels contained within the international standard (89SF).

	89SF μg/ml		
	IgG	IgA	IgM
4	4.1	1.2	1.4
14	27.8	1.9	1.2
23F	8.1	1.3	0.7

Table 2.1 The concentrations of serotype 4, 14 and 23F antibody in the international pneumococcal standard (89SF) for ELISA (supplied by the FDA).

A control serum was established (QC080806) by taking 20ml blood samples from previously immunised adults, pooling the serum and quantifying the IgG, IgA and IgM levels for serotypes 4, 14 and 23F against the 89SF standard curve and NIBSC control sera. Table 2.2 shows the geometric mean concentrations (GMC) for the new QC080806 control serum (+/- 2 standard deviations of the mean antibody concentration) for each serotype based on thirteen assay repetitions for each serotype and isotype. The assay acceptance criteria involved achieving a standard curve fit (r) of at least 0.97 and that the value for QC080806 fell within the +/- 2 SD range shown in table 2.2.

QC080606 µg/ml of antibody			
	IgG	IgA	IgM
4	1.24 (0.86-1.63)	0.44 (0.05-0.49)	0.27 (0.15-0.39)
14	20.55 (17.14-23.96)	478 (0.40-0.56)	1.76 (1.13-2.39)
23F	10.48 (8.74-12.22)	0.19 (0.18-0.21)	0.40 (0.26-0.55)

Table 2.2 Geometric mean (+/- 2 standard deviations) for QC080806.
An in-house control serum established from a pool of serum obtained from four adults who had previously received one or two doses of the Pnc7 vaccine.

IgG, IgA and IgM Antibodies specific for the capsular polysaccharides 4, 14 and 23F were detected using alkaline-phosphatase conjugates diluted 1:2000 for IgG and 1:1000 for IgA and IgM (section 2.1.3), and antibody concentrations were calculated in ng/ml from a four point, sigmoidal plot using Revelation™ softwear (ThermoLabs Inc., Basingstoke, UK). All the data were then subsequently converted to µg/ml for analysis.

2.11 T-cell stimulation and supernatant collection

PBMCs separated as described in section 2.4 and resupended to $2x10^6$cells/ml in 10%-HABS and 100µl of the suspension were added to the culture well giving a final well density of $2x10^5$ cells. The antigens for T cell stimulation were Tet (5µg/ml) dip (10µg/ml) CbpA and PspA at 2.5µg/ml (obtained from Dr James Paton, Adelaide, Au) and phytohemagglutinin (PHA) at 5µg/ml (Sigma, UK) were used in quadruplicate in round bottom culture plates, (Corning, UK). The cells were incubated with antigens for seven days at 37°C in 5%CO_2 and 95% humidity.

On day seven the supernatants were harvested from the wells and frozen at -80°C for subsequent detection of cytokines by ELISA. The remaining PBMCs were then harvested, washed in 10%-NBBS and then allowed to secrete IFNγ in the ELISpot assay described in section 2.12.

2.12 IFNγ ELISpot

The ELISpot for IFNγ secreting cells was carried out using the MABtech human IFNγ antibody kit as per the manufacturer's instructions (MABtech, Sweden). The kit contained anti-human IFNγ capture monoclonal antibody (Mab) (1-D1K), a biotinylated anti-human IFNγ detection Mab (7-B6-1) and a streptavidin-alkaline phosphatase enzyme conjugate. The same substrate and ELISpot plates were used as for the B-cell ELISpot, section 2.4. Briefly, the PVDF ELISPot plate (Millipore), was coated with 50µl/well of the anti-human IFNγ-Mab which was then incubated overnight at 4°C. The plate was then washed once with PBS and blocked with 10%-NBBS for at least 30 minutes at 37°C in 5%CO_2 and 95% humidity. The harvested cells from section 2.11, were added to the ELISpot wells in triplicate, at $1x10^5$ and $5x10^4$ cells/well and allowed to secrete IFNγ for 16-20 hours. Spots were enumerated using using an AID ELISpot reader ELR02 and software version 3.2.3. (Cadama Medical Ltd, Stourbridge, UK), using the preset IFNγ spot count settings from AID.

2.13 ELISA for IL-5 and IL-10 quantification

The concentrations of the cytokines IL-5 and IL-10 were quantified in the T cell culture supernatants using Becton Dickinson OptEIA ELISA antibody and buffer sets for detection of human IL-5 and IL-10, as per the manufacturer's instructions (BD Biosciences, Oxford UK). The T cell supernatants were defrosted and 50µl was added to duplicate wells, and the final concentration of cytokine was expressed in pg/ml.

2.14 CFSE dye dilution for quantification of B cell proliferation.

The cytoplasmic dye, 5-(and-6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE), was used to label PBMCs isolated at each time point after immunisation. The PBMCs were isolated

as in section 2.4 and resuspended to $2x10^6$cell/ml. Then 1ml of cell suspension was removed prior to CFSE labelling and kept as the unlabelled cell control and also for *ex vivo* FACS analysis prior to culture.

The remaining cells were diluted in PBS+EDTA+0.5%NBBS to give $1x10^6$ cells/ml. These cells were then labelled with a final concentration of 2μM CFSE by incubation at 37°C in 5% CO_2 and 95% humidity for 10 minutes. Following this incubation the reaction was quenched with ice cold PBS+EDTA+0.5%NBBS and washed at 2000 rpm (900xg) for 20 minutes. The pellet was resuspended in PBS+EDTA+0.5%NBBS and washed 2 times at 1800 rpm (700xg) for 15 minutes. Finally, the cells were resuspended in 1ml 10%NBBS, counted as in section 2.4.1 and then resuspended to $2x10^6$cells/ml and put into culture as in section 2.6.

Following 5 days of stimulation the cells were harvested for ELISpot as in section 2.7 and a small fraction was labelled with antibodies to IgD, IgM, IgG, CD27, CD19, and CD3 (see section 2.1.2 and section 2.8) The CFSE proliferation data was analysed by BD CellQuest™ software (see chapter 5).

2.15 Statistics

Comparison between individuals and time points was carried out using the Wilcoxon Signed Rank test and the Mann Whitney test in SPSS v12, GraphPad Prism V4 and Microsoft Excel.

Chapter 3: The T and B cell responses to a heptavalent pneumococcal conjugate vaccine at 12 months of age.

3.1 Abstract

To evaluate the response to a pneumococcal conjugate vaccine at 12 months of age, toddlers were immunised with a heptavalent pneumococcal protein-polysaccharide vaccine and blood samples obtained before and after immunisation. The B and T cell responses were evaluated by analysis of immunoglobulin and cytokines produced by PBMCs and surface phenotyping of the lymphocytes. There was a rise in frequency of diphtheria specific plasma cells following immunisation. *In vitro* culture with SAC+IL-2 revealed a rise in frequency of diphtheria and serotype 4 specific IgG memory B cells following immunisation. At different time points after immunisation there was variation in the number of memory B cells detected. The frequencies of polysaccharide specific memory B cells were higher at day 9 after immunisation than at day 11, while there was no difference in the number of the diphtheria specific cells at these two times.

There was a significant rise in serum IgG antibody following immunisation to serotype 4 and 23F but not to serotype 14, although pre-existing IgG levels were higher to serotype 14.

Toddler lymphocytes expressed a predominantly naïve phenotype ($CD27^-$ B cells and $CD45RA^+$ T cells). All of the toddler B cells expressed co-stimulatory molecules such as HLA-DR and CD38 and the T cells were $CD28^+$, suggesting a state of pre-activation and that they may respond rapidly even though they are naïve.

Pnc7 immunisation significantly increased the concentration of IL-5 and IL-10 in cell culture supernatants following *in vitro* stimulation of PBMCs with diphtheria toxoid. No such effect was seen with IFNγ-secreting cell frequency.

Prior nasopharyngeal carriage of pneumococcus (positive culture at d0) and increased risk of exposure to respiratory infections (exposure to siblings or peers in nursery) was associated with increased secretion of IL-5 and IL-10 by PBMCs cultured with diphtheria toxoid following Pnc7 immunisation. There was no change in the frequency of IFNγ-SC following immunisation and no difference between groups.

Conclusion

Toddlers responded well to a single dose of Pnc7 vaccine with plasma cells and memory B cells being generated in response to diphtheria toxoid and serotype 4. The cytokine response to the carrier protein appeared to be predominantly Th2 (IL-5) although the toddler's PBMCs were capable of secreting IFNγ. However, the time points chosen in the study may not have been ideal for optimal detection of plasma cells, memory B cells or for cytokine responses. While the data in this chapter have shown that it is possible to detect these B cell populations *in vitro* even in very young children, a number of questions have been raised. For instance, what are the optimal time points for plasma cell and memory B cell detection following immunisation?

3.2 Introduction

The heptavalent pneumococcal conjugate vaccine (Pnc7) is highly efficacious in children under the age of 2 years. However following immunisation with Hib and Men C conjugate vaccines at 2,3,4 months of age, the levels of serum IgG antibody have been shown to wane rapidly during the following 12-15 months (381). This was also shown to be true in infants primed with the Pnc7 vaccine, although the decline in antibody was serotype dependent (187). In some cases antibody concentrations may fall below the levels of detection by ELISA, yet boosting at a later date generates a rise in antibody concentration, indicating that priming for an immune memory response has occurred during the intervening time between priming course and booster dose.

The isolation and subsequent quantification of the B cells involved in the generation and maintenance of serum antibody may be of benefit in predicting future protective immunity induced by primary immunisation with glycoconjugate vaccines. This same process may also allow identification of individuals with pre-existing memory for the vaccine antigens. Antigen specific plasma cells have been isolated from the peripheral blood of human adults immunised with pneumococcal vaccines (106-108, 111, 113) and Hib vaccines (382). The presence of these cells in the circulation has also been demonstrated in pre-school children and toddlers, although direct comparisons with adults are few (113, 383). It has been suggested that toddlers predominantly generate non-secreting plasmablasts in response to pneumococcal polysaccharides. These cells migrate to the bone marrow before maturing into terminally differentiated plasma cells that secrete antibody for prolonged periods of time (256, 257, 354, 384-386). In order to detect non-secreting plasmablasts or pre-exisiting memory B cells, polyclonal stimuli are used to activate peripheral blood B cells, inducing the proliferative processes required for differentiation into IgG-antibody forming cells (IgG-AFC) (376, 387). The frequency of memory B cell derived IgG-AFC in the circulation of toddlers following immunisation with Pnc7, and comparison with adults, has not yet been established.

It is not only the B cell response that needs to be considered in response to protein conjugated polysaccharides. The success of the glycoconjugate vaccines in children under 2 years of age has been attributed to the recruitment of T cell help by the protein-carrier (in this case CRM197, a mutant of diphtheria toxoid). These $CD4^+$ T-helper cells (Th-cells) provide co-stimulatory help to B-cells responding both to the polysaccharide and also to the protein carrier. These signals include IL-5 that is produced by activated Th2 cells and mast cells and acts on B1 cells, stimulating proliferation and differentiation into AFC (285). However, the T cell help is affected by the cytokine environment in which the response to the vaccine antigens occurs (188, 189). This may be either the pre-existing cytokine environment or that induced by immunisation. The cells producing the cytokines may be non T cell and non B cell, therefore not specific for the

vaccine antigens at all. Previous studies have shown that priming immunisation schedules during the first 6 months of life can skew infant cytokine responses towards a Th2 phenotype (IL-5, IL-10 and decreasing IFNγ), that generates mainly IgM and IgG1 antibody (195, 388, 389). In older children and adults the response to immunisation is skewed towards a Th1 (IFNγ) or Th0 (IL-5, IL-10, IFNγ) response leading to IgG2 antibody. Factors which may affect the cytokines produced in response to immunisation include 1) the maturational stage of T cell development (390-392), 2) exposure to respiratory pathogens or nasopharyngeal carriage of commensal organisms (393, 394) and 3) maturational stage of accessory cell development such as natural killer (NK) cells (395) and defective maturation of dendritic cell IL-12 production (388). Exposure to *Bordetella pertussis* or immunisation with component pertussis vaccines during early infancy has been demonstrated to skew the T cell response towards a Th1 phenotype (396), while other childhood immunisations induce a Th2 phenotype (195, 388, 389) or a Th0 phenotype (189) which subsequent vaccination then drives toward either Th1 or Th2 phenotype depending on age. At 12 months of age, toddler T cell responses are already programmed toward a Th2 like phenotype in response to immunisations with tetanus toxoid and Hib. T cells from this age group have a steady state Th2 response and decreased IFNγ response. The new heptavalent pneumococcal conjugate has been shown to induce Th2 responses in adults, with respect to the carrier protein (373).

3.3 Aims

The main aim of this pilot study was to determine whether it would be possible to detect IgG-ASC specific for pneumococcal capsular polysaccharides in the peripheral blood of 12-month old toddlers following immunisation with a single dose of Pnc7. In particular it was of interest whether it would be possible to isolate these cell populations from the very small paediatric blood volumes of 4ml or less.

The secondary aim was to determine the frequency of interferon-gamma secreting cells (IFNγ–SC) as a measure of the Th1 cytokine response along with the levels of the Th2 cytokine IL-5 and regulatory cytokine IL-10 prior to and following immunisation in the same group of toddlers. Antibody data obtained from the main study(397) would then be compared to the cytokine data to see if there was an affect of Th2 skewing on antibody generation in response to immunisation.

3.4 Methods

3.4.1 Subjects and clinical procedures

A study was undertaken in the Oxford area in 12-month old toddlers. The aim of the study was to determine differences in serum IgG induced by immunisation with of Pnc7 in toddlers with or without risk factor for increased exposure to nasopharyngeal carriage of pneumococci (397). In the Salt *et al* study toddlers were grouped on the basis of risk factors for exposure to nasopharyngeal carriage of pneumococci during the first year of life. These risk factors were either a toddler's attendance of a day care facility or the presence of siblings in the family home, or both of these or neither of these.

For this study the grouping was simplified into two groups based on the increased risk (group 1) or reduced risk (group 2) of exposure to respiratory pathogens (Table 3.1).

The laboratory staff remained blinded to the toddler grouping until all of the study samples had been analysed. All of the participants received the same vaccine at 12 months of age.

Serum samples were collected prior to and 9-11 days following immunisation and a nasopharyngeal swab was obtained prior to immunisation. In order to study the B cell response in the same cohort ethical permission was sought from the Oxfordshire Research Ethics Committee (OxREC number CO2.005), to obtain 4ml of heparinised blood prior to and following

immunisation. Following this approval 5ml of blood was obtained from each child (1ml for ELISA and a maximum of 4ml for B cell studies, with the ELISA taking priority).

Therefore from a total of 160 toddlers enrolled and visited during the study, a small subset of 17 toddlers (14 paired samples), had sufficient sample for the successful detection of either *ex vivo* (plasma cells) or memory B cell derived IgG-AFC by ELISpot (table 3.1).

Cytokine (IL-5 and IL-10,) and IFNγ-SC (*in vitro* stimulated) responses were investigated in a further subset of twenty-nine 12-month old toddlers (table 3.1).

Blood volumes of less than 800μl were deemed to small to yield enough PBMCs for memory B cell culture and ELISpot, although they were sufficient for some T cell cultures since fewer cells were needed.

Study assay	Total no. toddlers tested	increased or reduced risk of exposure to a respiratory pathogen		isolation of a pneumococcus from nasopharyngeal swabs obtained prior to immunisation	
		Group 1 (increased)	Group 2 (reduced)	Positive	Negative
Plasma Cells	**7**	**n/a**	**n/a**	**n/a**	**n/a**
Memory B cells	**14**	**n/a**	**n/a**	**n/a**	**n/a**
IFNγ-SC	**29**	**17**	**12**	**17**	**12**
IL-5	**21**	**11**	**11**	**14**	**7**
IL-10	**21**	**11**	**11**	**14**	**7**

Table 3.1 The number of toddlers included in the analysis for each of the parameters tested in the study.
For each of the study assays the total number of toddler samples analysed is shown in the second column. For the cytokine assays the toddler samples were analysed for increased risk (group 1) and reduced risk (group 2) of exposure to respiratory pathogens. The cytokine data was also analysed based on whether nasopharyngeal isolates were culture positive or negative for detection of a pneumococcal serotype. (n/a = sample numbers were to small to perform the subgroup analyses for the B cell study).

3.4.2 B cell ELISpot for the detection of vaccine induced memory

PBMCs were isolated, as described in Chapter 2, section 2.4, from 12-month old toddlers (n=17) prior to and 9,10 or 11 days post-immunisation with a single dose of Pnc7.

The PBMCs were then either incubated directly in antigen coated ELispot wells or were cultured for 5 days in the presence of SAC+IL-2 as described in Chapter 2, sections 2.5-2.6.

The *ex vivo* elispot was carried out as in Chapter 2, section 2.5 and the data were expressed as IgG-AFC/10^6 PBMCs. For the memory B cell assay, following 5 days of *in vitro* stimulation with SAC+IL-2, the PBMCs were harvested, washed and seeded on to ELISpot plates ($2x10^5$ PBMCs/well), coated with PBS, tetanus toxoid, diphtheria toxoid and polysaccharides from the capsules of pneumococcal serotypes 4, 14 and 23F (See chapter 2 section 2.7).

Due to the small number of samples obtained for this part of the study it was not possible to determine the effects of nasopharyngeal carriage exposure on pre-existing memory B cell frequency. Statistical analysis was used to compare the difference in frequency of the IgG-ASC prior to and following immunisation for the *ex vivo* assay and also the memory B cell assay (Wilcoxon sign rank test for paired, non-parametric analysis). Analysis was also carried out on the effect of post immunisation time point (day 9, 10 or 11) on detectable memory B cell frequency specific for each of the antigens tested (Mann-Whitney U test for un paired, non-parametric analysis).

3.4.3 Determination of vaccine induced IFNγ-SC frequency and secreted cytokine concentrations

The frequency of IFNγ-secreting PBMCs was determined by ELISpot after 7 days of *in vitro* stimulation with tet, dip, CbpA and PspA (see chapter 2, sections 2.11 and 2.12). Tet was a positive, non vaccine related control antigen and dip was used to represent the CRM197 carrier protein response. CbpA and PspA were pneumococcal surface proteins to which toddlers may have been exposed during nasopharyngeal carriage of pneumococci.

The levels of the Th2 cytokine IL-5 and regulatory cytokine IL-10, in the cell culture supernatants were determined by ELISA as per manufacturers instructions (see chapter 2, section 2.13). Statistical analysis was used to compare the frequency of IFNγ-SC and IL-5 and IL-10

concentrations prior to and following immunisation (Wilcoxon sign rank test for paired, non-parametric analysis). Subgroup analysis was also carried out for the effect of exposure to respiratory pathogens and also whether carriage of a pneumococcus was detected prior to immunisation (Mann Whitney U test for un-paired, non-parametric analysis).

3.5 Results

3.5.1 A single dose of Pnc7 induced a rise in plasma cell frequency

Following immunisation a rise in the median frequency of spontaneously secreting IgG-AFC (plasma cells) was induced in response to the CRM197 carrier component of the vaccine (represented by dip). This was observed in 100% of the 12-month old toddlers 9-11 days after immunisation (table 3.2). A rise at day 9-11 was also seen in response to serotype-4 with 100% of participants responding to immunisation, though this was not significant, and to a lesser extent in response to serotype-14, but not in response to serotype-23F (table 3.2 and figure 3.1). The rise in frequency of IgG-AFC was highly significant for diphtheria toxoid (fig 3.1, p=0.006). Although there appeared to be a trend for a rise in frequency of IgG-AFC specific for the capsular polysaccharides (4, 14 and 23F,) this difference was not significant.

		median frequency of *ex vivo* IgG-AFC/10^6 PBMCs				
		tet	**dip**	**4**	**14**	**23F**
time point	**day 0**	**1**	**4**	**0**	**0**	**0**
	day 9-11	**0**	**20**	**5**	**5**	**0**
responders	**n=**	**3/7**	**7/7**	**7/7**	**5/7**	**1/4**
	%	**43%**	**100%**	**100%**	**71%**	**25%**

Table 3.2 The percentage of toddlers with an *ex vivo*, plasma cell response to a single dose of Pnc7. The data represent the median frequency of *ex vivo* AFC prior to (day 0) and following (day 9-11) immunisation of toddlers with a single dose of Pnc7. Also shown is the percentage of toddlers where a rise in frequency was seen following vaccination (% responding) with the actuall numbers of participants and responders in parenthesis.

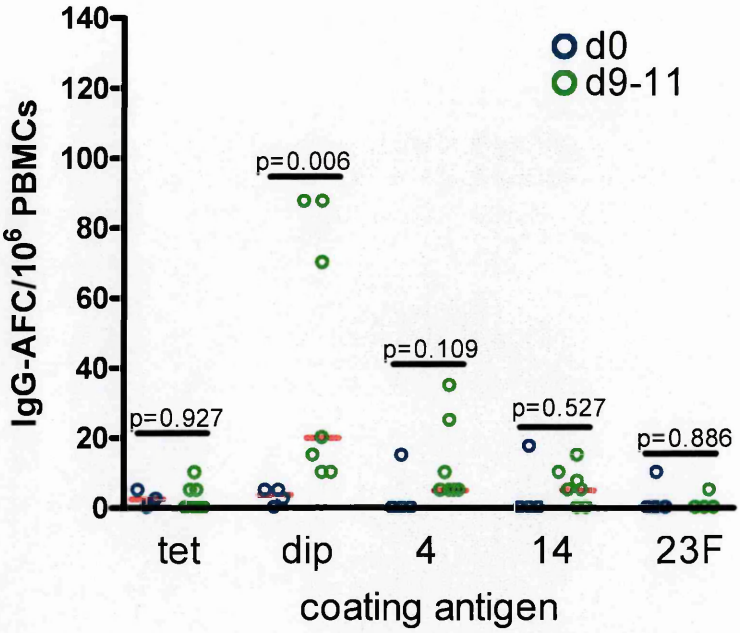


Figure 3.1 Spontaneously secreting IgG-AFC detected in the peripheral blood of 12-month old toddlers prior to (blue open circles) and 9-11 days after (green open circles) a single dose of Pnc7. PBMCs were isolated and incubated for 16 hours on ELISpot plates coated with tet, dip and serotype-4, 14 and 23F polysaccharides. Spots per well were calculated and the data were expressed as the number of antigen specific IgG-AFC/10^6 PBMCs.

3.5.2 Immunisation with Pnc7 did not lead to increased frequencies of Memory B cell derived total IgG-AFC.

The total frequency of all IgG-secreting cells isolated from the peripheral blood of toddlers was quantified by coating ELISpot wells with anti-human Ig and then detecting the AFC secreted antibody with an anti-human IgG-alkaline phosphatase conjugated antibody. Total IgG-AFC was used as a control for the in-vitro differentiation of memory B cells into IgG secreting plasma cells. The cut off frequency was ≥1000 IgG-AFC/10^6 cultured PBMCs. Samples were excluded if the frequency fell below this since the assay sensitivity for the low frequency polysaccharide specific memory B cells may have been insufficient. Figure 3.2 shows that it was possible to detect memory B cell derived IgG secreting cells in the peripheral blood of 100% of 12-month

old toddlers prior to immunisation. The lowest frequency detected was 2798 IgG-AFC/10^6 cultured PBMCs. Following immunisation there was no significant alteration in the frequency of total IgG-secreting cells. The range in total IgG-AFC/10^6 cultured PBMCs, both before and after immunisation, was wide (2798-62,500 total IgG secreting cells before and 7500-40,000 9-11 day after immunisation).

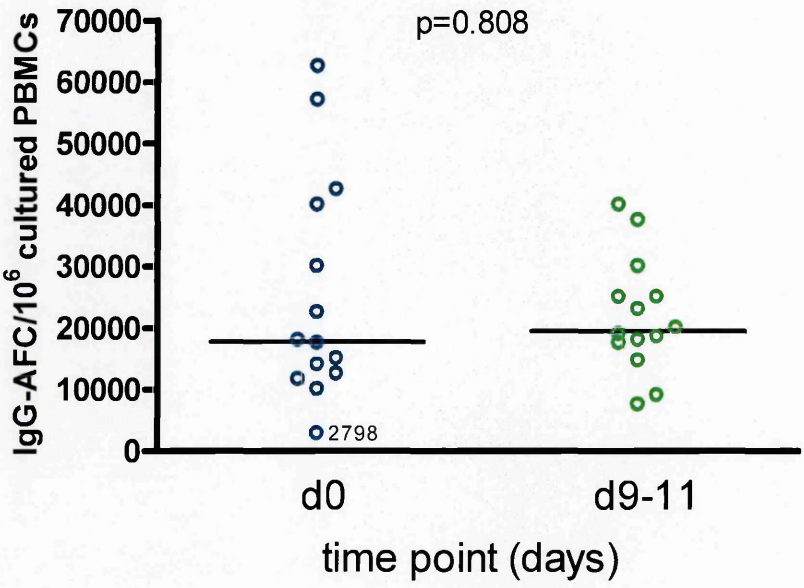


Figure 3.2 The frequency of total-IgG-AFC in the peripheral blood of toddlers prior to (d0) and following (d9-11) a single dose of Pnc7.
PBMCs were isolated and stimulated with SAC+IL-2 for 5 days. The cells were then harvested and washed 3 times in 10%-NBBS before being seeded (at concentrations of $2x10^4$, $2x10^3$, $2x10^2$ cells/well), into anti-human Ig coated ELISpot wells. The cells were then left to secrete IgG overnight at 37°C in 5%CO_2 and 95% humidity. Following development of the spots, the data were expressed as the median number of IgG-AFC/10^6 cultured PBMCs. The effect of immunisation on the frequency of total-IgG AFC was calculated in a non-parametric, paired analysis using the Wilcoxon signed rank test.

3.5.3 A single dose of Pnc7 increased the frequency of antigen specific memory B cell derived AFC.

Tetanus toxoid (tet) was used as a positive control to check that *in vitro* stimulation of PBMCs drove the differentiation of antigen specific memory B cells in to plasma cells. There were detectable numbers of tet specific IgG-AFC in 93% of toddlers prior to immunisation and this was unaltered following administration of the Pnc7 vaccine (figure 3.3). The median number of tetanus toxoid specific cells detected prior to immunisation was 12 IgG-AFC/10^6 cultured PBMCs while following immunisation this fell to 8 IgG-AFC/10^6 cultured PBMCs (p=0.808).

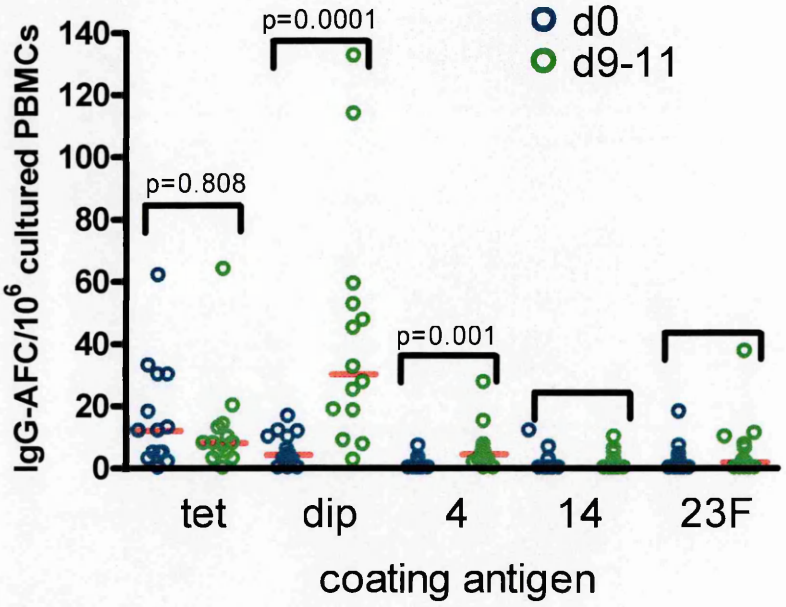


Figure 3.3 The frequency of antigen specific IgG-AFC, derived from memory B cells from the peripheral blood of toddlers.
PBMCs were isolated on day 0 (blue open circle) and then days 9-11 (green open circle) following immunisation with a single dose of Pnc7. The PBMCs were stimulated with SAC+IL-2 for 5 days, and then harvested, washed 3 times in 10%-NBBS and seeded (at 2x10^5cells/well), into ELISpot wells coated with tet, dip, and serotypes 4, 14 and 23F polysaccharides. The cells were incubated overnight at 37°C in 5%$CO_2$and 95% humidity. Following development of the spots, the data were expressed as the number of IgG-AFC/10^6 cultured PBMCs. The Wilcoxon signed rank test was used to calculated the significance of differences in the median cell frequency between day 0 and day 9-11.

The frequency of memory B cells specific for the vaccine related antigens were determined prior to immunisation. The CRM_{197} carrier response was determined using diphtheria toxoid as the *in vitro* antigen and, before immunisation, 79% of the toddlers had detectable diphtheria toxoid specific memory IgG-AFC, (median of 4 IgG-AFC/10^6 cultured PBMCs). Pre-existing memory B cell frequency specific for the polysaccharide antigens was much lower. Only 29% of toddlers had detectable memory B cells specific for serotypes-4 and 14 and 43% for serotype-23F (table 3.3). This translated into median spot counts of zero for all three polysaccharides.

Following immunisation, memory IgG-AFC specific for diphtheria and serotype-4 increased significantly in frequency by days 9-11 (fig 3.3), with 100% of toddlers showing a boosted memory response to diphtheria and 79% for serotype-4 (table 3.3). There was no significant alteration in the frequency of IgG memory to serotypes-14 and 23F. The percentage of toddlers with detectable IgG-AFC following immunisation was higher for all of the antigens at days 9-11, though the actual number of individuals showing an increase in frequency (% responders) did not change for serotype-14 and showed only a marginal rise for serotype-23F (table 3.3).

The low level of detection for some of the serotypes suggested that perhaps the timing of the blood samples was too late and that the peak in circulating antigen specific memory B cell had been missed. Therefore the data were split to show the frequency of cells detected on days 9 (n=4), 10 (n=7/8) and 11 (n=7) following immunisation (fig 3.4).

It is clear from figure 3.4 that there was no significant alteration in the detection of the IgG-AFC to the positive control (fig 3.4a tetanus) at any of the time points, while for diphtheria toxoid the frequency of IgG-AFC remained significantly elevated above baseline at each of the time points (fig 3.4b). For each of the capsular polysaccharide antigens there was a trend to decreasing frequency of IgG-AFC as time post immunisation increased with day 9 yielding the highest rate of detection (fig 3.4c-e), although this was only significant for serotype-4 (fig 3.4c).

		median frequency of IgG-AFC/10^6 cultured PBMCs			
		dip	4	14	23F
day 0	median frequency	4	0	0	0
	n-pre	11/14	4/14	4/14	6/14
	% pre-existing memory	79%	29%	29%	43%
day 9-11	median frequency	30	4	0	2
	n-post	14/14	12/14	5/14	9/14
responders	n-resp	14/14	11/14	4/14	7/14
	%	100%	79%	29%	50%

Table 3.3 The frequency of memory B cell derived IgG-AFC in the peripheral blood of toddlers prior to and following a single dose of Pnc7.
The median frequency of IgG-AFC are shown for day 0 and day 9-11, expressed as the median IgG-AFC/10^6 cultured PBMCs. Underneath this is the number of individuals (n-pre) with pre-existing memory IgG-AFC at day 0 plus the percentage. At day 9-11 the number of toddlers with detectable IgG-AFC (n-post) is shown. Finally the overall percentage of toddlers with a rise in frequency of IgG-AFC to each of the antigens (% responders) and also the absolute numbers (n-resp) following immunisation with Pnc7 vaccine is shown.

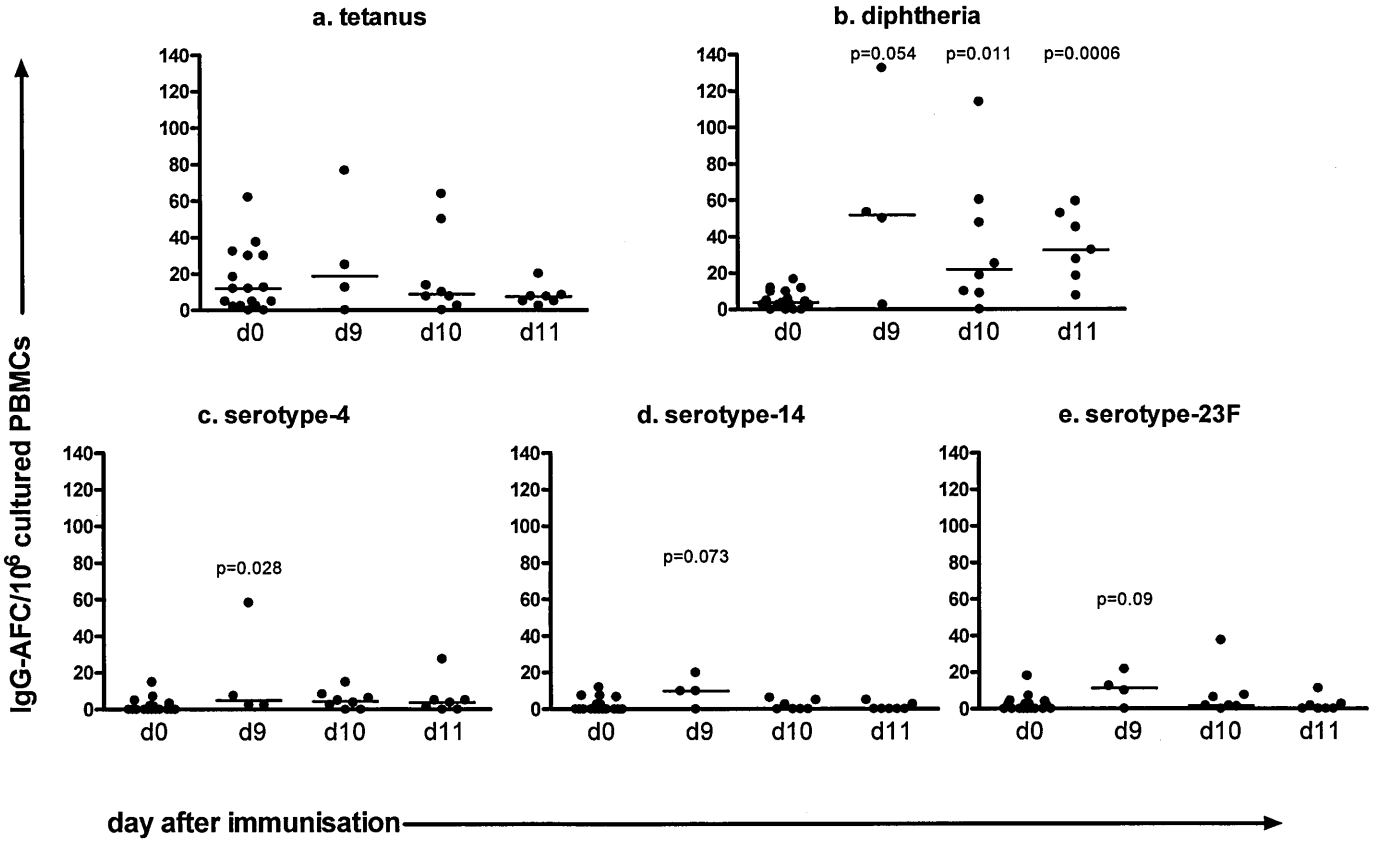


Figure 3.4 The effect of post vaccination time point on the detection of memory B cell derived IgG-AFC from the peripheral blood of toddlers.
The data represent the frequency of antigen specific IgG-AFC derived from memory B cells following *in vitro* stimulation of PBMCs with SAC+IL-2 for 5 days. The cells were harvested, seeded in well coated with a) tetanus, b) diphtheria, and c-e) serotype 4, 14 and 23F polysaccharides. The data are expressed as the number of IgG-AFC/106 cultured PBMCs with the median shown by the line for each time point prior to (d0) and after immunisation (d9-11) with Pnc7.

3.5.4 Pnc7 immunisation induced a rise in pneumococcal capsule specific serum IgG antibody.

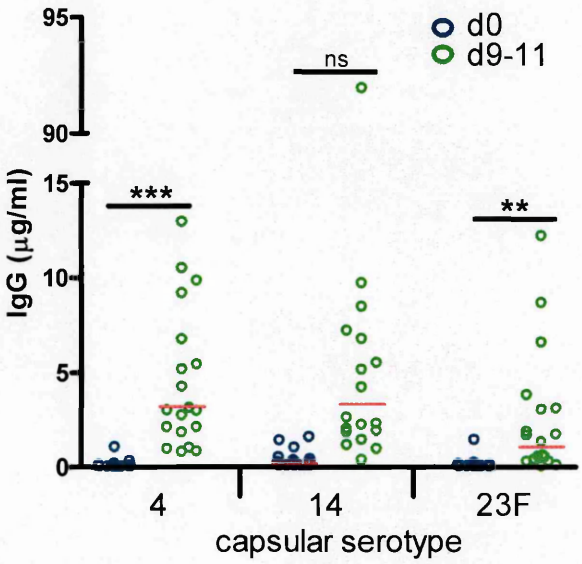


Figure 3.5 Serotype capsular polysaccharide specific IgG in the serum of 12 month old toddlers prior to and following a single dose of Pnc7.
Serum IgG antibody was quantified by the ELISA protocol as described in Chapter 2, section 2.10. This data represents a subset of 18 individuals from the larger study of 160 subjects, the results of which were published recently by P. Salt *et al* (397). The individuals included here were the same individuals included in the memory B cell study. The data represents the serum concentration of IgG (µg/ml) at day 0 (blue circles) and day 9-11 post immunisation (green circles) with a single dose of Pnc7. The red bar represents the GMC with. ***p=<0.0001, **p=0.0067, ns=not significant, p=0.1134.

Prior to immunisation there were barely detectable levels of IgG to any of the three serotypes with GMCs for serotypes 4, 14 and 23F of 0.068µg/ml (0.01-1.06), 0.201µg/ml (0.114-0.354) and 0.07µg/ml (0.04-0.118) respectively (figure 3.5). These levels increased significantly for serotype 4 (p=<0.0001), and 23F (p=0.0067), following immunisation while in response to serotype 14 the rise in GMC of IgG was not significant (p=0.1134, fig.3.5). The post immunisation levels of IgG were similar for each of the serotypes while the actual rise in IgG specific for serotype 4 was greater than for serotype 14 which was greater than that induced by

23F. There were no correlations between antibody levels and memory B cell frequencies at any time point for any of the three serotypes.

3.5.5 A single dose of Pnc7 had no effect on the phenotype of toddler B and T cells.

Toddler PBMCs were isolated and the cells labelled for expression of CD3, CD19/CD20 along with activation and co-stimulatory markers CD28, CD38, CD69 and CD80 as described in chapter 2, sections 2.1.2 and 2.8. The mean expression of B-cell activation and memory markers are show in fig.3.6a-b, while the general characteristics of the $CD3^+$ T cells are shown in figure 3.6c.

The percentage of $CD19^+$ and $CD20^+$ B cells was identical (fig.3.6a-b), which allowed for more versatility in combinations of antibodies used for further analysis of phenotypes. Toddler B cells were predominantly naïve ($CD27^-$) but positive for the MHC class II molecule HLA-DR (fig.3.6b). The B cell expression of the co-stimulatory marker CD80 and the activation marker CD69 was also low. Most toddler B cells were $CD38^+$, and the mean $CD38^+CD19^+$ B cell population increased following immunisation, though not significantly.

The T cell phenotype (fig 3.6c) shows that almost all of the T cells expressed CD28 and CD38. A small percentage of the T cells express the IL-2 receptor (CD25) and 50% expressed CD45RA (a marker of naïve T cells), and 10% express CD45RO (a memory T cell marker). The activation marker CD69 was not detected. Thus the toddler T cells were predominantly naïve but expressed co-stimulatory markers (CD28 and CD38). Therefore immunisation of toddlers with a single dose of Pnc7 had no observable effect on the mean percentages of the B cell or T cell populations (fig.3.6a-c).

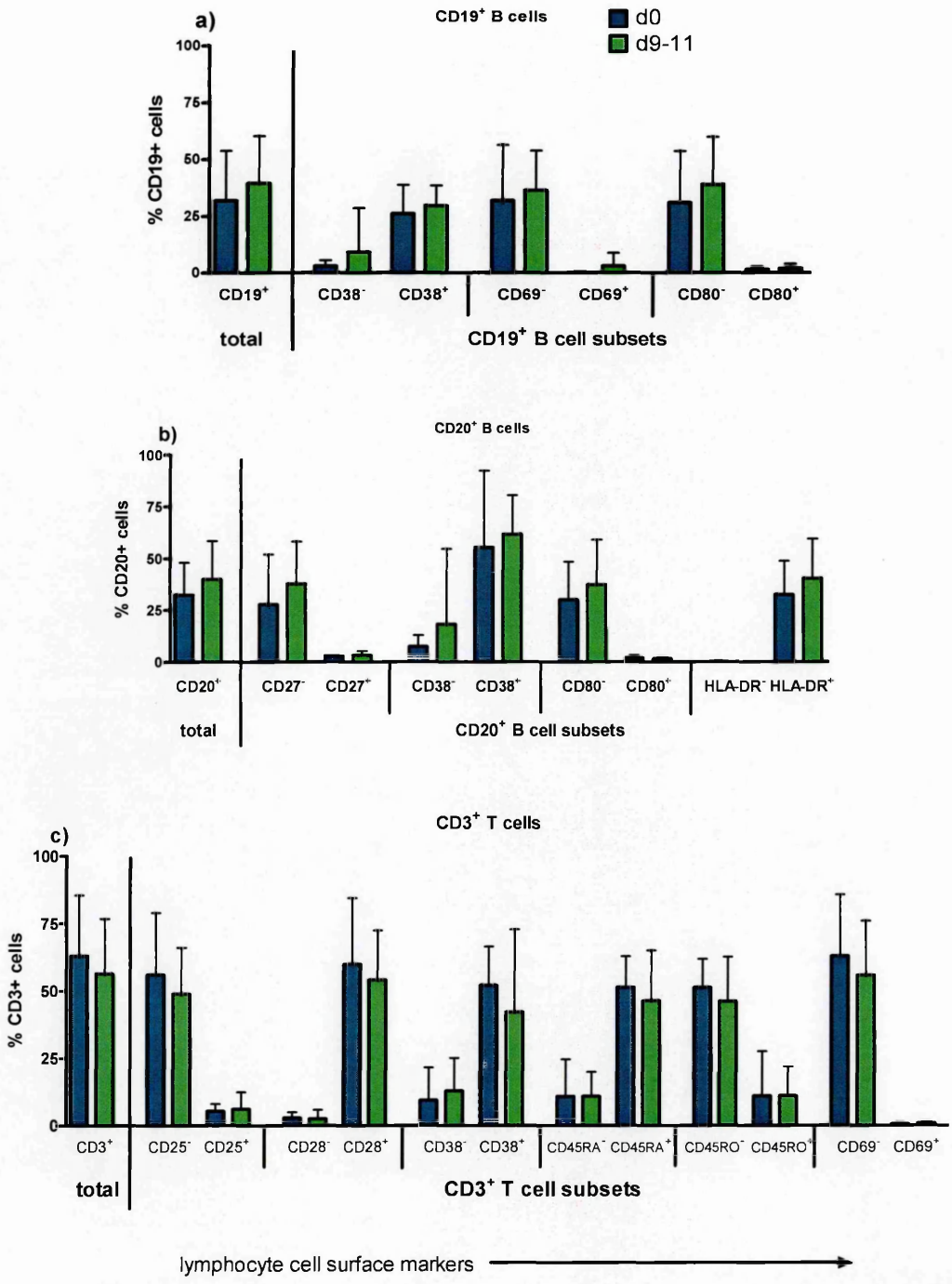


Figure 3.6 The phenotype of peripheral blood B and T cells isolated from toddlers prior to (blue) and 9-11 after a single dose of the Pnc7 vaccine (green).
B cells were gated on either CD19 or CD20 surface marker expression (total). The within the CD19/CD20$^+$ populations of B cells the percentage expression of CD27, CD38, HLA-DR, CD80 or CD69 was determined (a-b). The T cells were gated based on the expression of CD3. The percentage of CD3$^+$ T cells expressing CD25, CD28, CD38, CD45RA/RO, and CD69 was then quantified (c). The bars represent the mean percentage surface expression within the CD19/CD20$^+$ or CD3$^+$ populations plus the 95% confidence intervals.

3.5.6 Reduction in spontaneous IFNγ secretion by PBMCs isolated following immunisation with Pnc7.

The frequency of IFNγ-SC was quantified following *in vitro* stimulation of PBMCs isolated prior to and 9-11 days after immunisation of toddlers with Pnc7. A group of twenty-nine 12-month old toddlers were included in the analysis but the limited size of blood samples obtained meant that not all of the culture antigens were used for all of the subjects and the number of paired samples was much lower than the 29 individuals recruited into the study (figure 3.7).

There was a wide variation in the frequency of IFNγ-SC between individuals in response to stimulation with all of the antigens before and after immunisation (figure 3.7).

In vitro stimulation of PBMCs with tet, dip (vaccine CRM197 carrier protein related antigen), PspA and PHA induced higher frequencies of IFNγ-SC than was seen with medium alone figure 3.7 ($p=<0.0001$), while there was no difference in the frequency of CbpA induced IFNγ-SC compared to medium alone ($p=0.109$).

Following Pnc7 immunisation there was a significant decline in the spontaneous secretion of IFNγ in the medium only PBMC cultures (fig. 3.7a, $p=0.017$) but no change in IFNγ-SC frequency following PBMC stimulation with specific antigens or PHA (fig.3.7a-b).

Therefore immunisation did not alter the IFNγ response to stimulation with dip (the CRM197 carrier protein related antigen), nor to the other antigens or PHA but there was a reduction in spontaneous IFNγ secretion in the medium only cultures.

Table 3.4 shows the proportion of toddlers where immunisation with Pnc7 increased the propensity of stimulated PBMCs to secrete IFNγ. There was a rise in IFNγ-SC frequency in 28%-53% of toddlers following *in vitro* culture of post immunisation versus pre immunsation PBMCs.

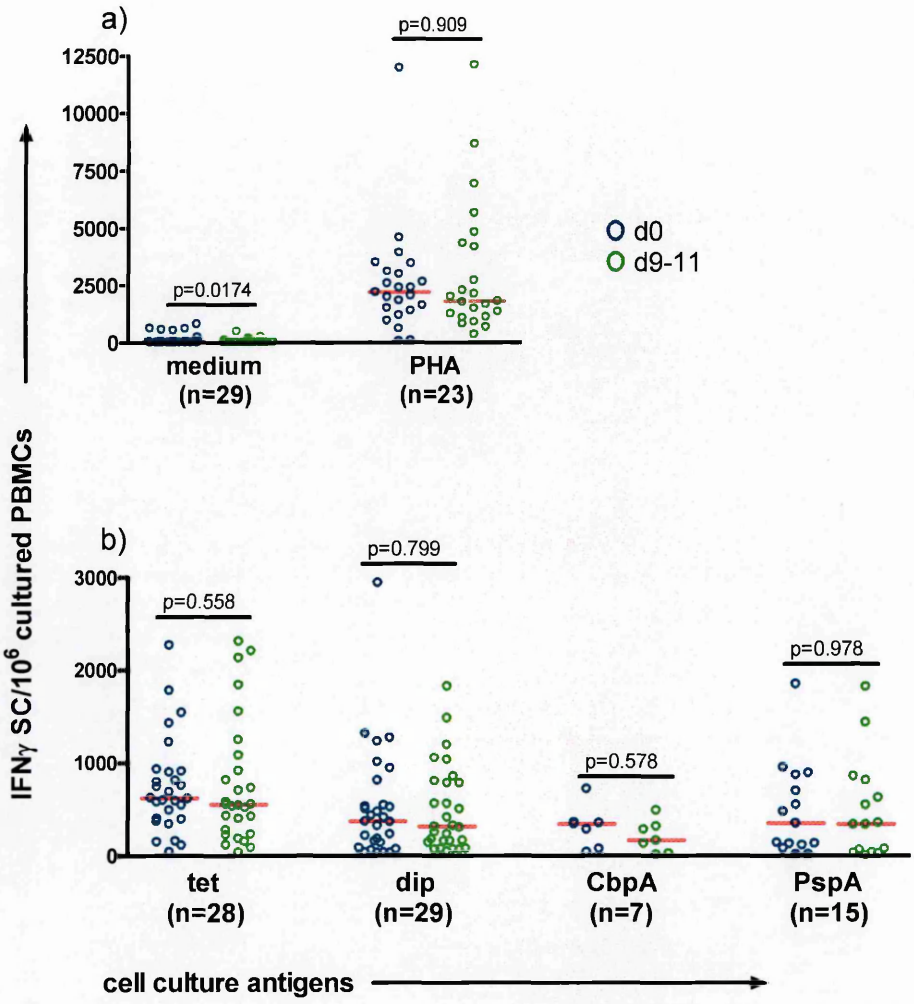


Figure 3.7 The effect of immunisation on the frequency of spontaneous and antigen specific IFNγ-SC. PBMCs isolated prior to immunisation (blue) and 9-11 days after immunisation with a single dose of Pnc7 (green) were stimulated for seven days with medium or antigens and three days with PHA. The resulting cells were then harvested and seeded into anti-IFNγ-Mab coated ELISpot wells where they were allowed to secrete IFNγ for 16 hours. IFNγ-SC were then enumerated and the data expressed as IFNγ-SC/10^6 cultured PBMCs. Fig 3.7a shows the frequency of IFNγ-SC obtained from unstimulated PBMCs (medium alone), and polyclonaly stimulated PBMCs (PHA). Fig.3.7b shows the frequency of IFNγ-SC obtained following stimulation of PBMCs with tet, dip, CbpA and PspA. The red lines represent the median number of IFNγ-SC.

		antigens used for *in vitro* stimulation of PBMCs isolated 9-11 days after immunisation with Pnc7					
cytokine	**responders**	**medium**	**tet**	**dip**	**PspA**	**CbpA**	**PHA**
IFNγ-SC	**%**	28%*	36%	48%	29%	53%	48%
	n=	**8/29**	**10/28**	**14/29**	**2/7**	**8/15**	**11/23**
IL-5	**%**	10%	40%	80%*			
	n=	**2/21**	**8/20**	**16/20**			
IL-10	**%**	19%	29%	60%*			
	n=	**4/21**	**6/21**	**12/20**			

Table 3.4 The percentage of toddlers where a single dose of the Pnc7 vaccine lead to an increased propensity of *in vitro* stimulated PBMCs to make IFNγ, IL-5 or IL-10 9-11 after immunisation with Pnc7.
PBMCs were isolated following immunisation and stimulated *in vitro* with tet, dip, PspA, CbpA, PHA or medium only. IFNγ-SC frequency was enumerated by ELISpot and concentrations of IL-5 and IL-10 in the culture supernatants were measured by ELISA. The data shown are the percentage responders (%) with actual numbers of toddlers tested and responding (n=). *$p<0.05$.

3.5.7 Increased IL-5 and IL-10 secretion by PBMCs isolated following immunisation with Pnc7.

Determination of IL-5 and IL-10 cytokine levels in cell culture supernatants was only possible in response to *in vitro* stimulation of PBMCs with tet and dip (figure 3.8 and table 3.4).

The concentration of IL-5 induced following *in vitro* stimulation of PBMCs with tet or dip was higher than that of IL-10. PBMCs stimulated with tet or dip secreted more IL-5 and IL-10 than those cultured in medium alone (fig 3.8a-b). Levels of IL-5 and IL-10 were highest in response to *in vitro* stimulation with tet although immunisation of toddlers with Pnc7 did not alter this. However, the Pnc7 vaccine contains CRM197, a mutant diphtheria toxoid, and following Pnc7 immunisation the levels of IL-5 and IL-10 secreted following *in vitro* stimulation of PBMCs with dip increased significantly (IL-5, $p=0.027$ and IL-10, $p=0.016$ respectively, fig.3.8a-b) with 80% and 60% responders respectively (table 3.4).

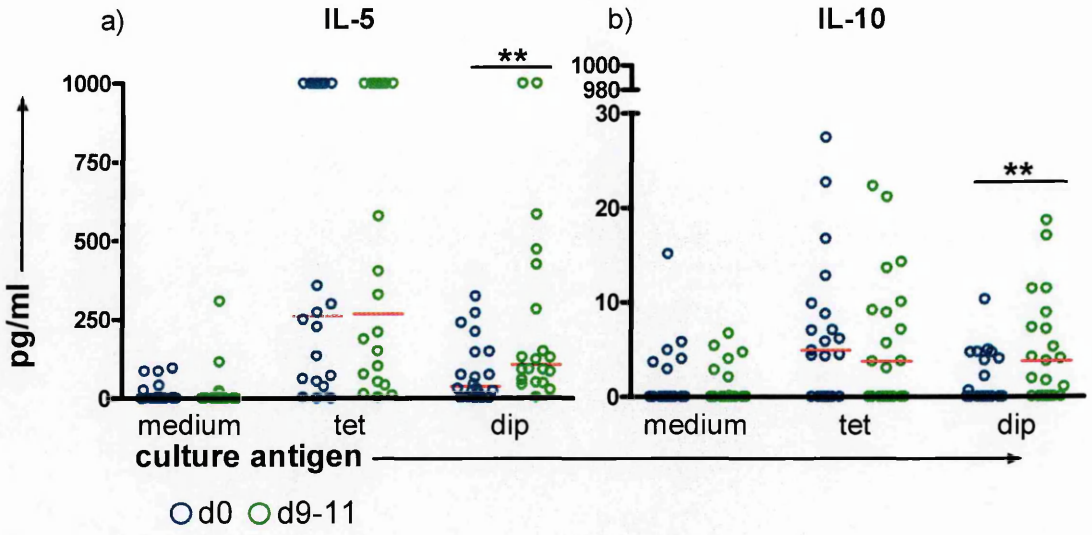


Figure 3.8 IL-5 and IL-10 secretion by PBMCs isolated before and after immunisation of toddlers with a single dose of Pnc7.
PBMCs were isolated from toddlers on day 0 (blue open circles) and on day 9-11 after immunisation (green open circles). The PBMCs were then cultured for seven days in the presence of medium only, tet or dip. The level of IL-5 (a) or IL-10 (b) in the supernatant was measured by ELISA and is presented in pg/ml with the median concentration shown as a red bar.

3.5.8 The effect of post immunisation blood sample time point on frequency of IFNγ-SC

When the d0 samples were grouped based on the timing of the post vaccination sample (d9, 10 or 11), it was possible to see that there was no difference in the propensity of PBMCs to secrete IFNγ at day 0 between the groups or between each group compared to the study population as a whole (fig.3.9a-c).

Following immunisation the frequency of IFNγ-SC cells induced by *in vitro* stimulation of PBMCs with tet and dip was unaffected by the timing of the post immunisation sample (fig.3.9e-f). However, PBMC cultured with medium alone showed significantly higher frequencies of IFNγ-SC at day 11 than at day 9 ($p=0.024$, fig 3.9d) but there was no significant difference between d0 and d9, 10 or 11. This data suggests that frequencies of IFNγ-SC may rise above baseline at a time later than day 11.

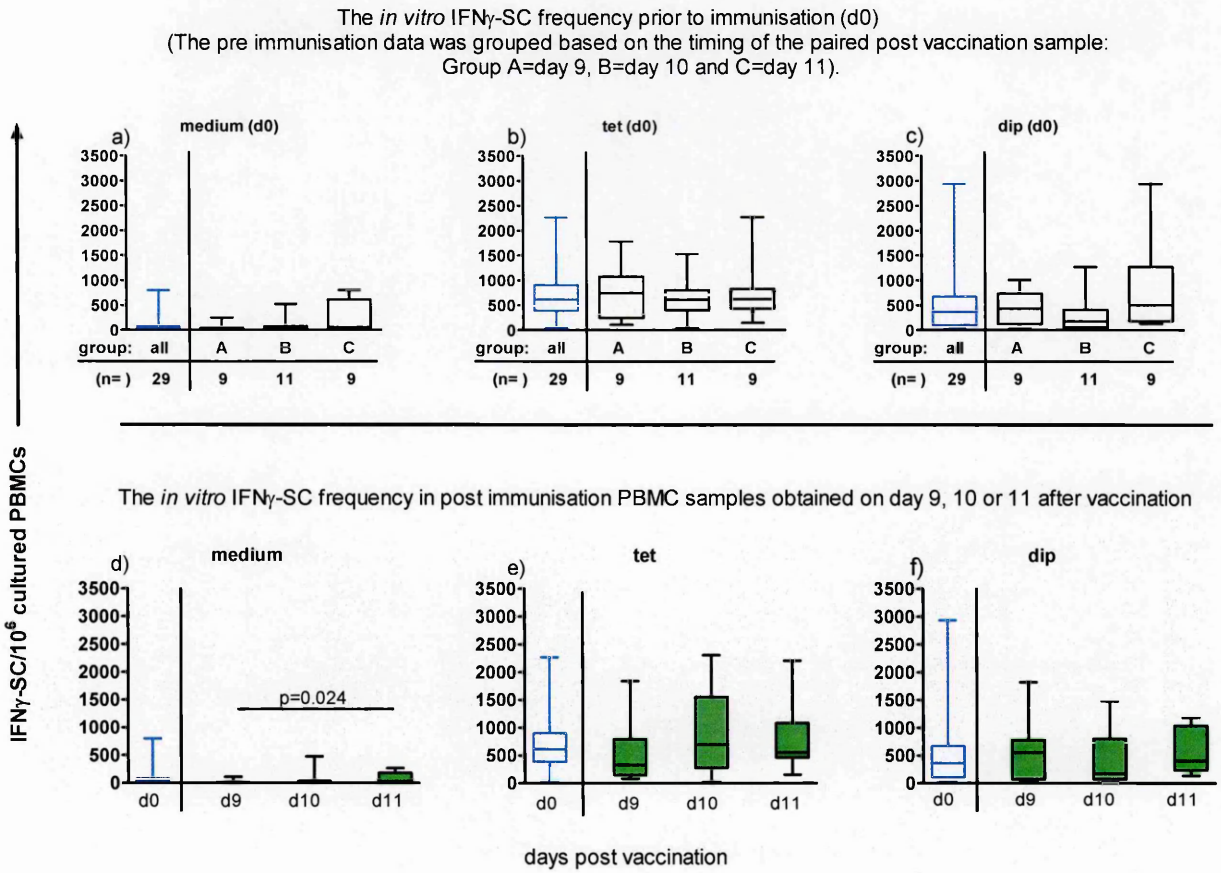


Figure 3.9 The effect of the post immunisation time point (day 9, 10 or 11) on detection of IFNγ-SC. PBMC were isolated prior to (d0) and on day 9, 10 or 11 following immunisation with Pnc7.

The PBMCs were then stimulated *in vitro* with medium only, tet or dip for seven days. For analysis the d0 and d9-11 samples were paired and then grouped based on the post vaccination time point. So the d0 groups were A= paired with d9, B=d10 and C=d11. The d0 (baseline) IFNγ-SC frequencies were then compared between the groups (A, B, C) and with the overall (all) d0 frequency (3.9a-c). The d0 frequencies for all subjects (blue) were then used in the comparison of baseline IFNγ-SC frequency with that at each post vaccination time point (d9, 10 or 11, green) in 3.9d-f.

3.5.9 Exposure to respiratory pathogens had no effect on the IFNγ-SC frequency but enhanced diphtheria toxoid induced IL-5 and IL-10

The toddlers recruited into this study were grouped according to risk factors for exposure to respiratory pathogens. These risk factors were i) presence of siblings in the household and ii) attendance of daycare. For the purposes of this thesis analysis the toddlers were grouped as follows: Group 1 = increased risk of exposure to respiratory pathogens and Group 2 = reduced risk (no siblings or daycare). The frequency of IFNγ-SC and also the concentrations of IL-5 and IL-10 in the supernatants of the PBMC cultures were compared between the groups both before and after immunisation with Pnc7 (fig 3.10).

Immunisation with Pnc7 had no effect on the frequency of IFNγ-SC following *in vitro* stimulation of PBMCs with medium, tet or dip (fig.3.10a-c). There was also no difference in IFNγ-SC frequency between group 1 and group 2.

The concentrations of IL-5 (fig.3.10d-f) and IL-10 (fig.3.10g-i), in the PBMC culture supernatants, was similar between group 1 and group 2. This was true both before and after immunisation. Within group 2 however, following immunisation with Pnc7 there was a significant rise in the concentrations of IL-5 ($p=0.027$) and IL-10 ($p=0.016$) in the culture supernatants following *in vitro* stimulation with diphtheria toxoid than was seen prior to vaccination (fig.3.10.f+i).

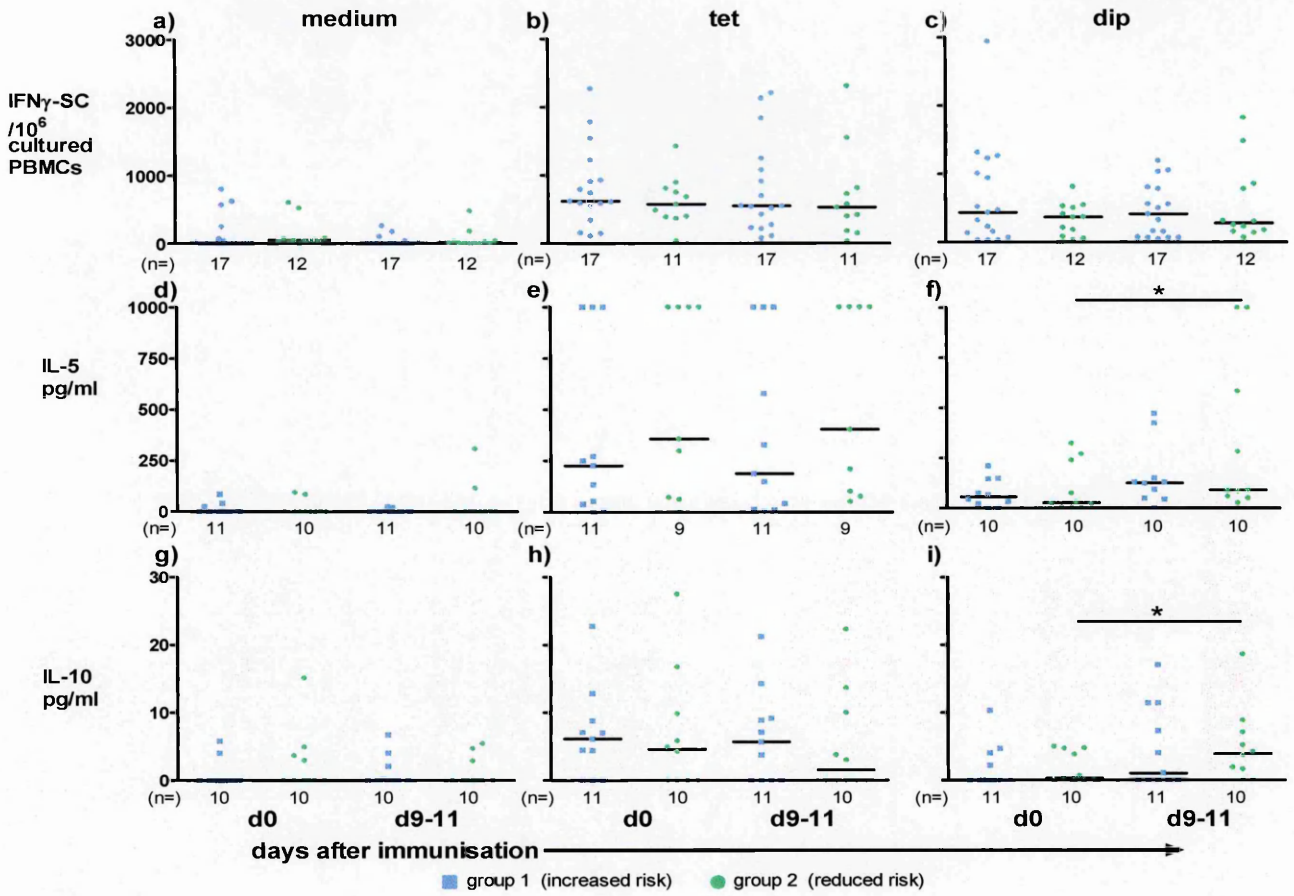


Figure 3.10 The effect of exposure to respiratory infections on the subsequent production of IFNγ-SC or IL-5 and IL-10 secretion in PBMC cultures. Samples were obtained on d0 and d9-11 following Pnc7 immunisation. The toddlers were grouped for increased risk of exposure to respiratory infection (group 1 ■) or reduced risk of exposure to respiratory infections (group 2 ●). PBMCs were stimulated *in vitro* for seven days with medium only (3.10a-g), tet (3.10b-h) or dip (3.10c-i). IFNγ-SC are expressed per 10^6 cultured PBMCs and IL-5 and IL-10 as pg/ml. The bar represents the median. *p=<0.05.

3.5.10 Carriage of pneumococci prior to Pnc7 immunisation increases the diphtheria toxoid specific IL-5 secretion by PBMCs.

As part of the larger study, 159 toddlers were shown to have had cultures, positive for pneumococcus isolated from nasopharyngeal swabs obtained prior to immunisation. For this portion of the study the percentage of positive versus negative cultures is shown in table 3.5. The main observation from this data was that more children were positive for pneumococcal carriage (59-70%) than were negative for carriage at the time of immunisation (30-43%).

This data shows that there were similar proportions of pneumococcal culture positive and negative toddlers in each of the cytokine analysis groups. The IFNγ-SC frequencies for the two groups (culture positive or negative,) are shown in figure 3.11(a-d) along with the concentrations of IL-5 (fig. 3.11e-h) and IL-10 (fig. 3.11i-l).

	Antigen used for *in vitro* stimulation							
	medium		**tetanus**		**diphtheria**		**PHA**	
culture (+/-)	+	-	+	-	+	-	+	-
IFNγ-SC	**59%**	**41%**	**57%**	**43%**	**59%**	**41%**	**61%**	**39%**
IL-5	**67%**	**33%**	**70%**	**30%**	**65%**	**35%**	**62.5%**	**37.5%**
IL-10	**65%**	**35%**	**70%**	**30%**	**67%**	**33%**	**59%**	**41%**

Table 3.5 Percentage of toddlers with detectable pneumococcal carriage prior to immunisation with Pnc7.
Nasopharyngeal swabs were obtained from all of the toddlers in the main study (397) and the percentage of cultures positive or negative for the detection of pneumococci was established. This data represents the proportion of toddlers included in each of the cytokine-antigen analysis groups, that were either positive or negative.

Following *in vitro* stimulation of the PBMCs isolated both before and after Pnc7 immunisation there was a higher concentration of IL-5 than IL-10 present in the culture supernatants. This was true following stimulation with tetanus (fig.3.11f+j), diphtheria (fig.3.11g+k) and PHA (fig.3.11h+l). There were no significant differences between the culture positive and culture negative groups at either time point (d0 or d9-11). However immunisation with Pnc7 did result in

a significant increase in the IL-5 secretion by PBMCs isolated from the culture positive group following *in vitro* stimulation with diphtheria toxoid (fig.3.11g, p=0.017). The frequency of IFNγ-SC obtained from the same *in vitro* PBMC cultures was similar in the culture positive and culture negative groups (fig.3.11.a-d). Therefore, only the IL-5 response of PBMCs appeared to be affected by prior exposure to pneumococci.

3.5.11 Toddler rather than adult PBMCs made a greater IL-5 and IFNγ response to diphtheria toxoid stimulation.

In order to determine whether baseline levels of IL-5, IL-10 and IFNγ-SC were affected by age the baseline levels of IL-5, IL-10 and frequency of IFNγ-SC in the toddlers were compared with that found in a group of five young adults (figure 3.12a-c). There was no difference in IL-10 concentration between toddlers and adults, irrespective of the stimulatory antigen used (fig.3.12b). The concentrations of IL-5 were lower in the adult group than in the toddler group, particularly in response to stimulation with diphtheria toxoid (fig.3.12a, p=0.013). There were also differences in the frequency of tetanus and diphtheria toxoid specific IFNγ-SC between the two age groups with toddlers having significantly higher IFNγ-SC frequencies. No such difference was seen in response to stimulation of PBMCs with PspA or PHA.

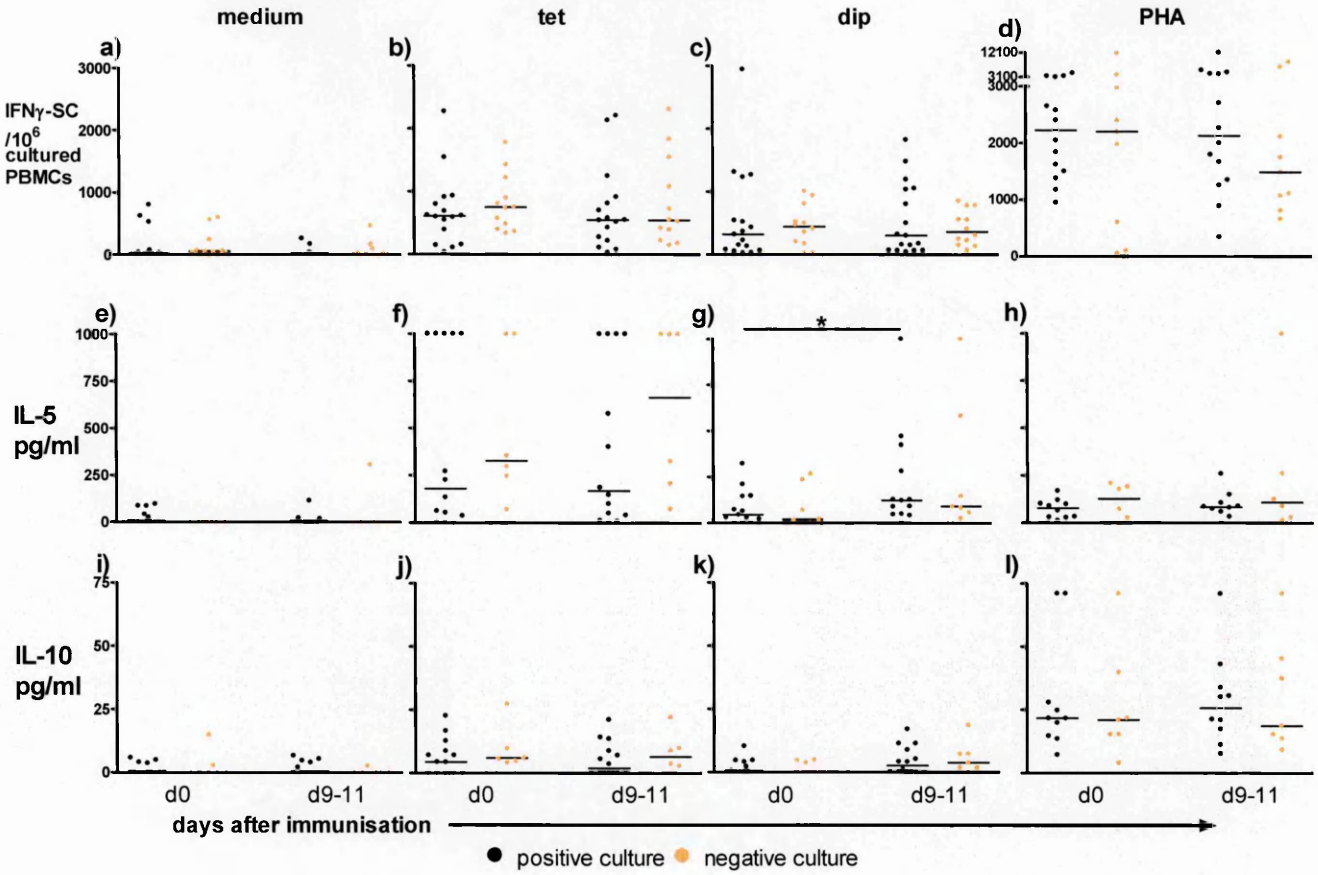


Figure 3.11 IFNγ, IL-5 and IL-10 response of PBMCs isolated before and after immunisation of toddlers with a single dose of Pnc7.
The toddlers were grouped according to pneumococcal culture positive (black) or culture negative (orange) nasopharyngeal isolates obtained prior to immunisation. PBMCs were isolated from toddles in these groups on d0 and d9-11 after immunisation. PBMCs were stimulated *in vitro* for seven days with medium only (a, e, i) tet (b,f,j) dip (c, g, k) and PHA (d, h, l). The supernatant were collected and IL-5 (e-h) and IL-10 (i-l) concentrations were measured by ELISA (pg/ml) while the cells were harvested and seeded onto anti-IFNγ coated ELISpot wells and allowed to secrete IFNγ overnight. The frequency of the IFNγ-SC were expressed as IFNγ-SC/10^6 cultured PBMCs (a-d).

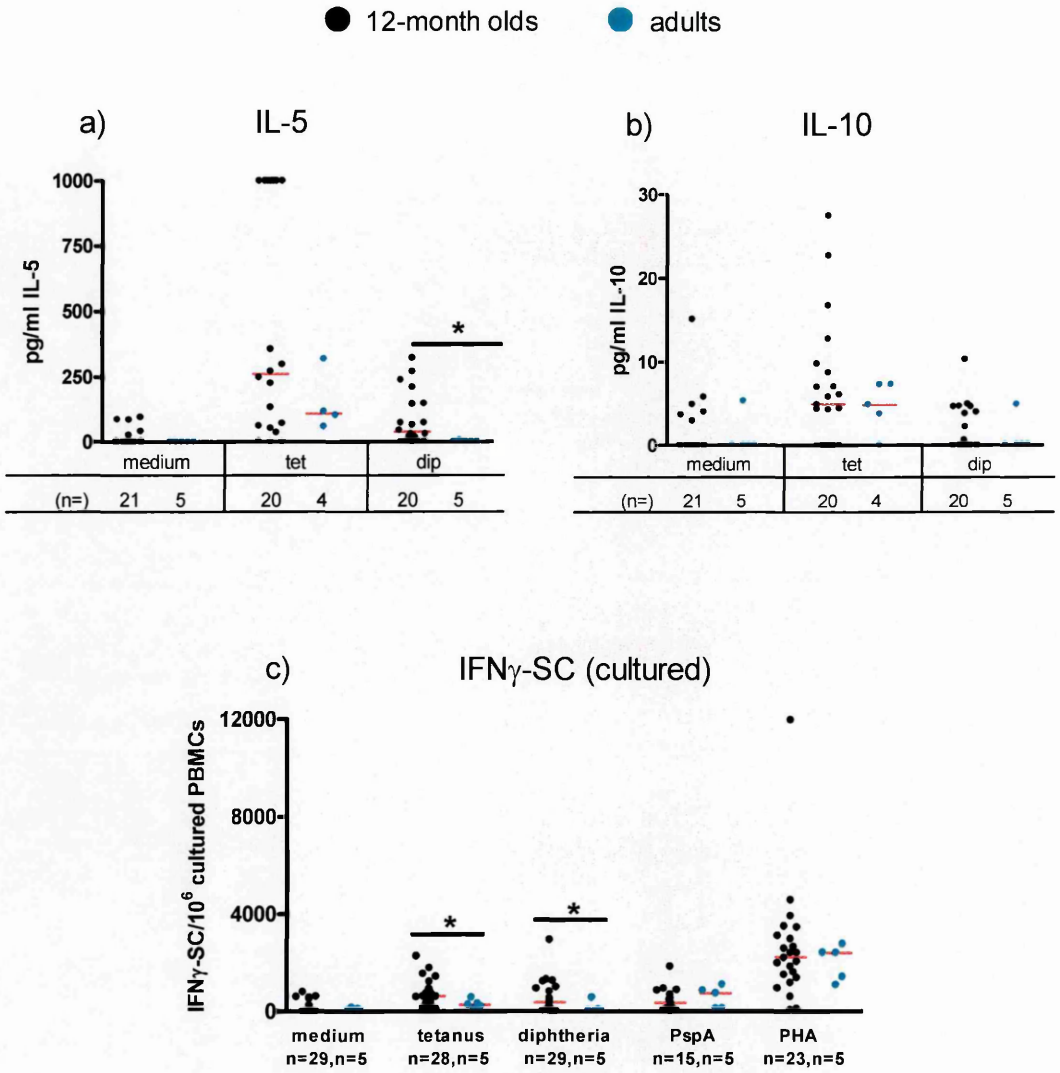


Figure 3.12 Inducible IL-5 and IL-10 concentrations and IFNγ-SC frequency from *in vitro* cultures of PBMCs obtained from toddlers and adults prior to immunisation with Pnc7.
PBMCs were isolated and stimulated for seven days with either medium only, tetanus or diphtheria toxoid, PspA and PHA. The supernatants were collected and IL-5 (a) and IL-10 (b) concentrations were quantified by ELISA. The cultured cells were harvested and allowed to secrete IFNγ on to anti-IFNγ coated ELISpot wells for 16 hours. The frequency was then determined and expressed as the number of IFNγ-SC/10^6 cultures PBMCs (c).

3.5.12 Correlation between serum IgG and frequency of memory B cells

Serum IgG concentrations specific to each of the capsular polysaccharides (4, 6B, 9V, 14, 18C, 19F and 23F), were quantified as part of the larger study and the data published recently (397). The data for the three serotypes used in the B cell studies (4, 14 and 23F) were compared with the memory B cell frequency. No correlation was found either prior to or following immunisation except for serotype 23F. At day 0 there was a very weak, negative correlation between serum IgG and memory B cell frequency ($r=-0.4977$, $p=0.042$).

3.5.13 Correlation between serum IgG concentrations and inducible IFNγ-SC frequency

For PBMCs isolated prior to immunisation with Pnc7 and stimulated with PHA there was a weak correlation between the IFNγ-SC response and post immunisation levels of IgG specific for serotype 4 ($r=0.4472$, $p=0.032$). Stimulation of the same PBMC samples with tetanus toxoid generated an IFNγ-SC response that weakly correlated with serum IgG specific for serotype 9V ($r=0.4545$, $p=0.017$) and 23F ($r=0.4329$, $p=0.021$). There was also a weak association with post immunisation levels of IgG specific for 14 ($r=0.4028$, $p=0.034$) and 23F ($r=0.5547$, $p=0.0022$). Following Pnc7 immunisation, stimulation of PBMCs with tetanus toxoid generates IFNγ-SC frequencies that were weakly associated with post immunisation IgG specific for serotype 19F ($r=0.3846$, $p=0.04$). Stimulation with diphtheria toxoid induced IFNγ-SC frequencies that correlated with IgG specific for serotype 14 ($r=0.4048$, $p=0.029$).

3.5.14 Correlation between serum IgG concentrations and inducible IL-5 and IL-10

As with IFNγ-SC the correlations between serum IgG and IL-5 or IL-10 were weak.

The main relationships found involved spontaneously induced IL-5. For example the levels of IL-5 found in unstimulated cultures prior to immunisation negatively correlated with baseline serum IgG specific for serotype 6B (r=-0.614, p=0.004,) and 9V(r=-0.5549, p=0.011). Baseline IL-5 from unstimulated cultures also negatively correlated with post immunisation IgG specific for serotype-6B (r=-0.7922, p=<0.0001).

Following immunisation the IL-5 produced in unstimulated cultures still showed the same correlations with serum IgG. Overall, spontaneously secreted IL-5 negatively correlated with IgG levels while antigen induced production of IL-5 positively correlated with serum IgG levels.

The levels of IL-10 spontaneously secreted in unstimulated PBMC cultures did not correlate with serum IgG concentration. However, IL-10 induced in response to PBMC stimulation with PHA correlated with IgG prior to immunisation for serotype 6B (r=0.5283, p=0.029).

Prior to immunisation, stimulation of PBMCs with tetanus toxoid induced levels of IL-10 that positively correlated with serotype 9V specific IgG (r=0.4940, p=0.027), 19F (r=0.4619, p=0.035), and 23F (r=0.4985, p=0.021).

Following immunisation, stimulation of PBMCs with tetanus toxoid resulted in IL-10 levels that weakly correlated with IgG specific to serotype 9V (r=0.6223, p=0.0034), 23F (r=0.5062, p=0.019), 23F (d9-11) (r=0.5100, p=0.018.) While IL-10 produced by PBMCs stimulated with diphtheria toxoid correlated with IgG specific for 23F (pre and post immunisation (r=0.4395, p=0.046; r=0.4420, p=0.045 respectively).

3.6 Discussion

3.6.1 A single dose of Pnc7 induced elevated frequencies of both plasma cells and memory B cells

The data from this study show that it was possible to detect both plasma cells *ex vivo* and memory B cell derived IgG-AFC isolated from small (≤4ml of whole blood), paediatric whole blood samples.

Pnc7 immunisation induced a significant rise in plasma cell frequency in response to the carrier related protein (diphtheria toxoid, fig.3.1). There was also a rise in frequency of polysaccharide specific plasma cells specific for serotypes 4 and 14, though these were not significant), but no such rise was seen in response to 23F (fig.3.1).

The frequency of memory B cell derived AFC also rose significantly in response to diphtheria toxoid and serotype 4 after immunisation with Pnc7, but no increase was seen in response to serotype 14 and 23F.

There was no correlation between the memory B cell frequency and post immunisation antibody level. However, the largest fold rise in antibody titre was seen in response to serotype 4 and this was the only serotype with a significant rise in frequency of memory B cells. The antibody data obtained from this subset of individuals followed the trends described in the paper by Salt *et al* 2007 (397) on the complete cohort of 160 12 month old toddlers. Here they found that serotype 4 was the most immunogenic (100% of toddlers achieved titres >0.2μg/ml), followed by serotypes 14 and 23F (99% and 81% of toddlers achieving IgG titres >0.2μg/ml).

The serum antibody response and the frequency of memory B cells, although not correlated, show similar patterns of response. The significant rise in memory B cells following immunisation in response to serotype 4 mirrors the antibody response at the same time point. However, there was minimal polysaccharide specific memory detected in peripheral blood before immunisation. So where did the pre-existing antibody derive from? This result argues for persistence of long-lived plasma cell maintenance in the bone marrow of these 12 month old toddlers from prior exposure to pneumococci or following diphtheria toxoid immunisations (258, 354, 385, 398). However, if this is the case then why were the responses to the polysaccharides from serotypes 14 and 23F not as efficient? One reason for the discrepancy may be a lack of nasopharyngeal carriage of these serotypes prior to immunisation? However, the carriage data from this and other studies show that infact serotypes 23F and 14 are detected more frequently in carriage isolates than serotype 4 (59, 79, 92, 397). The numbers in this pilot study were far too small to complete a subset analysis

for the influence of prior nasopharyngeal carriage on the B cell response, but Salt *et al* (397) did report differences in the magnitude of the antibody response for some of the vaccine serotypes in toddlers who had been exposed to carriage and those who had not. From data presented here it is only possible to conclude that differences in the immunogenicity of the capsular polysaccharides themselves are responsible for the variation in the immune responses.

The frequency of antigen specific memory B cells peaked significantly above baseline at day 9 for the polysaccharide antigens but in response to diphtheria this elevation remained by day 11 post immunisation. It was not possible to carry out a similar analysis on the plasma cell response since the number of individuals was too small. However, higher frequencies of spontaneously secreting cells have been observed during the first seven days following antigen challenge. Studies in the 1980's showed that following immunisation of adults with pneumococcal polysaccharide vaccine there was a rise in spontaneously secreting IgG-AFC during the first week after immunisation, followed by a rise in antibody(106-108, 261). However, it has not been shown when memory B-cell derived IgG-AFC peak in the peripheral blood of toddlers or adults after immunisation with a glycoconjugate vaccine.

The dynamics of the extrafollicular and germinal centre reactions suggest that antigen specific antibody secreting cells appear in the peripheral blood from day 6 post immunisation, however spontaneously secreting plasma cells tend to disappear from the periphery by day 8-9 (254, 259, 354, 399). Thus the timing of the post immunisation samples in this pilot study in relation to previously described kinetics suggests that the frequency of spontaneously secreting plasma cells (fig.3.1), was probably on the wane and this may be the sole reason for the low frequencies detected.

A population of non-secreting plasma cell precursors (plasmablasts) has also been described exiting the lymphoid tissues after antigen exposure (254, 257, 354, 400). These cells migrate to the bone marrow under the influence of CXCR4-CXCL12 interactions were they mature into

terminally differentiated plasma cells and secrete antibody in the long term (348, 350, 354, 357, 401). These cells are highly proliferative and will differentiate into mature immunoglobulin secreting cells after *in vitro* stimulation with SAC+IL-2 (400-402). Therefore between the *ex vivo* plasma cell and *in vitro* stimulated memory B cell assay it is probable that these data represent the early response mixture of mature plasma cells and non-secreting plasmablasts which can evolve from naïve B cells, MZB, FO B cells and memory B cells(385).

The germinal centre reaction reaches a peak of B cell numbers by days 10-14 after immunisation (401) therefore it is likely that some of the AFC detected at days 9-11 in this study are derived from a newly generated memory B cell response.

There were no correlations between *in vitro* derived IgG-AFC and the concentration of IgG antibody detected in the serum. One reason for this lack of correlation may be the mixed source of plasma cells, secreting antibody in the short term and plasmablasts that are non secreting.

Figure 3.13 shows a scheme which describes the kinetics of the B cell subsets and their relationship to the serum antibody being secreted during the early response to a single immunisation with Pnc7, a T cell dependent vaccine.

Following immunisation there are two waves of B cell proliferation and differentiation. One is extrafollicular and independent of direct T cell interaction and the second is dependent on T cells and germinal centre formation (figure 3.13). The blue boxed area shows how the samples obtained in this pilot study relate to the kinetics of the different B cell response.

Spontaneously secreting plasma cells are the product of the extrafollicular response and are short lived, peaking in frequency by day 7 (green filled curve), thus at the time points in this study the frequency of PC was probably waning, based on findings in previous studies (106-109, 145, 259, 261).

When PBMCs were stimulated *in vitro* with SAC+IL-2 the cells differentiating into AFC were most likely non secreting plasmablasts produced during the initial extrafollicular response (red

line). Plasmablast migration has been shown to continue for some days (up to five weeks in mice (259)), following immunisation. So at the time points in this study, although spontaneously secreting plasma cells were disappearing from the peripheral blood, accounting for the low AFC frequency in the *ex vivo* ELISpot, the *in vitro* stimulation of PBMCs enabled continued detection of IgG-AFC by day 11 after immunisation.

The germinal centre reaction involves the recruitment of T cells by FDC expressing specific antigen, allowing co localisation of B cells and T cells for appropriate co-stimulation and help. By days 9-11 after immunisation the germinal centre activity is reaching its peak and the resulting cells exiting the germinal centre include newly generated plasmablasts (solid and dotted red line) and memory B cells (dotted blue line). The kinetics of these cells are uncertain and it is unlikely that there will be spontaneous antibody secretion, hence the waning, antigen specific *ex vivo* response. However the germinal centre plasmablasts and memory B cells induced, by SAC+IL-2, to differentiate in to AFC will contribute to the frequency of cells detected in the memory B cell ELISpot.

The antibody response is represented by the green line and is initially produced by the wave of extrafollicular, short lived plasma cells (green filled curve) present in peripheral blood in the first week after immunisation. In a secondary response IgG, IgA and IgM antibody accumulates in the serum, peaking by day 15 and remaining elevated for up to 1 month before declining gradually in the absence of re-stimulation (187). Plasmablasts migrate to the bone marrow where they mature into long-lived plasma cells so, *in vivo*, do not contribute to the initial antibody response.

Thus the lack of correlation between serum antibody on days 9-11 and the *in vitro* memory B cell frequency is because these B cells are not responsible for producing the antibody.

To better understand the kinetics of the response to the pneumococcal conjugate vaccine in more detail a pilot study in a small group of adults was undertaken to look at the frequency of spontaneously secreting plasma cells and also memory B cell derived IgG-AFC (Chapter 4.)

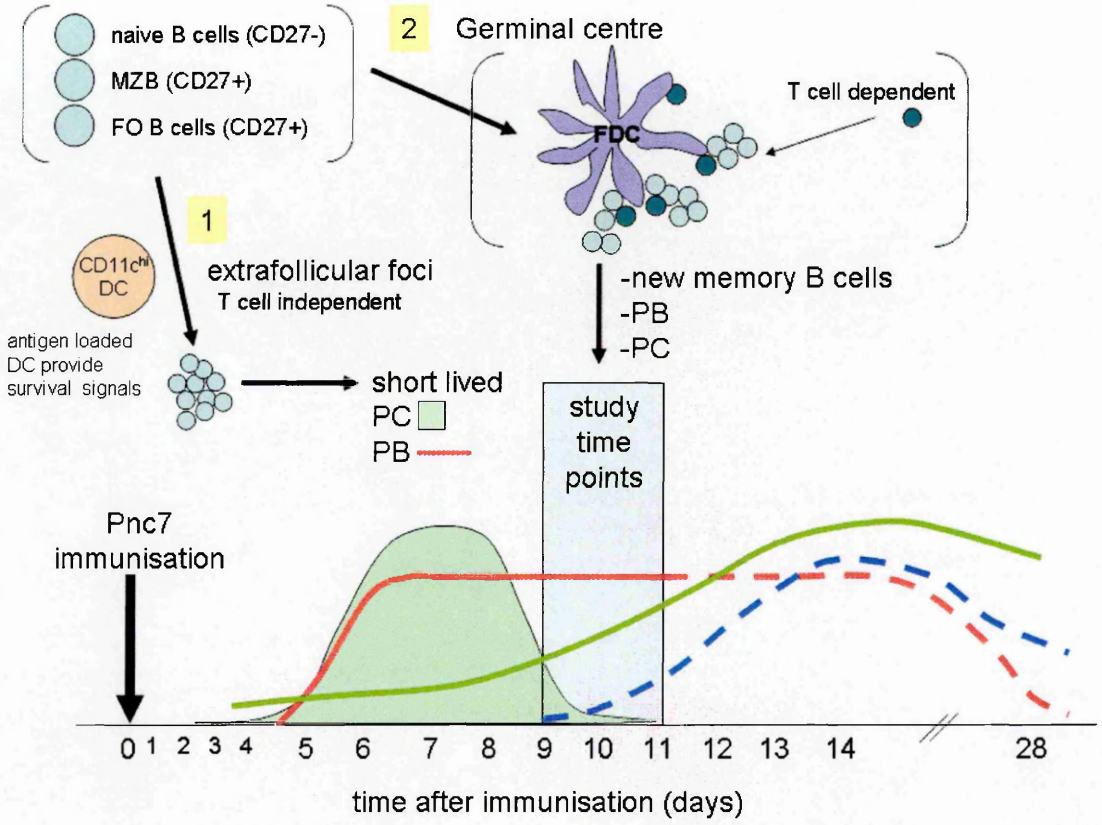


Figure 3.13 The source of serum antibody produced during the initial immune response to a single dose of Pnc7 vaccine.
In the initial phase of the immune response to a TD glycoconjugate vaccine naïve B cells, marginal zone B cells (MZB) and follicular B cells (FO) undergo rapid extrafollicular proliferation and differentiation in response to B cell receptor cross-linking (1). The survival of these B cells is aided by signals provided by dendritic cells (DC). The result of this initial B cell response is the production of short lived, antibody secreting plasma cells (PC) and non-secreting plasmablasts (PB). The kinetics of the PC response is shown by the green, filled curve and the proposed PB kinetics by the red line. The lime green line shows what happens to the serum IgG response during the first month after immunisation. Most of this initial antibody is secreted by the short lived plasma cells and peaks by day 15 after immunisation. In the first few days, low avidity antibody is produced by MZB but by the second week, FO B cells differentiate into higher avidity IgG secreting PC. T cells are recruited into the response by the carrier protein in the vaccine and this leads to the formation of germinal centres (2), from which arise memory B cells and non secreting PB. The blue line shows the proposed kinetics for detection of the memory B cell response *in vitro* while the PB frequency in peripheral blood may eventually wane in the absence of antigen, while serum IgG is maintained by PB differentiation into long lived PC in the bone marrow. The blue boxed area shows the time points covered by the samples in this pilot study.

3.6.2 Peripheral blood lymphocytes in toddlers are predominantly naïve in phenotype

The B cells isolated from 12 month old toddlers were mainly CD27- and the T cells CD45RA+ showing that the immune system was still immature. B cells expressing CD38 were predominant in toddler peripheral blood. CD38 is differentiation marker that is up or down regulated during different stages of B-cell development, and is highly expressed in terminally differentiated plasma cells. It has previously been described that 6 days after secondary immunisation with tetanus toxoid that there was an increase in $CD38^+$ cells in peripheral blood in mice and humans (262). This was not seen in this study, perhaps because the time points of the blood draws were too late. There was a small population of CD80+ B cells both before and after immunisation with no change in the percentage. It has previously been demonstrated that children aged 2-20 months express low to negative levels of co-stimulatory molecules such as CD80 on the surface of their resting B cells as is seen in adults (403). However a subset of memory B cells has been described in adult humans that express CD80 even when resting. It is suggested that these $CD80^+CD27^+$ B cells may be involved in rapid secondary responses as they secrete large amounts of switched immunoglobulins (361, 404). Within the $CD20^+$ B cell population in this study the expression of CD27 and CD80 were quite similar and it may be that these were the same cells (fig 3.6b), although double staining was not possible at this point to confirm this. If these cells were $CD80^+CD27^+$ then is would show that the toddlers possessed such a population of rapidly responding, switched memory B cells that may be involved in early responses to infection or immunisation.

3.6.3 Toddler T cell responses are skewed to Th2

In this study the T cells isolated prior to immunisation could be induced to secret IFNγ, IL-5 and IL-10. When compared to adult baseline levels the frequency of IFNγ-SC cells induced by stimulation with diphtheria and tetanus toxoid was significantly higher in the toddler group. This

is likely to be the result of more recent tetanus and diphtheria immunisations in the toddlers. There were no age related differences in the frequency of IFNγ-SC stimulated by PspA or the mitogen PHA. A number of studies have shown that IFNγ responses increase with age and number of vaccine doses, reaching adult levels by 12 months of age (391, 392).

There were equivalent levels of inducible IL-10 in both adult and toddler PBMC cultures while IL-5 levels in cultures of toddler PBMCs were higher than in adult cultures. Together with the IFNγ-SC results, this suggests that prior to immunisation, toddler PBMCs were already more skewed toward a Th0 (IFNγ and IL-5) cytokine phenotype than were adults.

Studies in humans of all ages have a revealed a gradual maturation of the neonatal T cell response through to toddlers and young children that is increasingly skewed towards a Th1 response, but with stable levels of Th2 cytokine expression (390-392, 395). This increasing production of IFNγ is thought to be related to the gradual acquisition of a memory $CD4^+$ T cell phenotype ($CD45RO^+CD45RA^-$) with age (390). The gradual rise in the Th1 responsiveness of toddlers T cells may account for the non-polarised cytokine pattern seen here compared to adults.

3.6.4 The Response to a single dose of Pnc7 vaccine

Immunisation of toddlers with a single dose of Pnc7 only enhanced the amounts of IL-5 and IL-10 (fig 3.6) produced in response to *in vitro* stimulation with diphtheria toxoid (the representative antigen for the CRM197 carrier protein in Pnc7 vaccine). There was no alteration in the frequency of IFNγ-SC and infact the spontaneous secretion of IFNγ-SC from unstimulated PBM cultures decreased following vaccination with Pnc7 (fig 3.5). Previous studies of tetanus immunisation in neonates followed through to 12 months of age showed that although infants made IFNγ responses they were short lived and waned by 6 months of age, even using polyclonal stimuli in the cell cultures. Th2 cytokine responses remained stable and boosted with re-immunisation (389). A similar effect was seen in response to hepatitis B vaccine, Hib-CRM197 conjugate vaccine and measles vaccine (195, 388, 405). The data from this study also show that

immunisation did not boost the *in vitro* IFNγ response but did boost the *in vitro* IL-5 response to diphtheria toxoid.

The Pnc7 vaccine contains aluminium hydroxide as an adjuvant and this in itself can alter cytokine responses, generally toward increased IFNγ production by activated phagocytic cells or NK cells (373). Adjuvants used in a murine study of pneumococcal conjugate vaccines found similar effects with QuilA and with CpG plus dimethyl dioctadecyl ammonium bromide. In both cases a Th1 profile of cytokines was induced along with IgG2a and IgG2b (Th1 associated in mice) (406). In a previous study of adults immunised with pneumococcal conjugate vaccines containing seven polysaccharides conjugated to tetanus toxoid or one containing four polysaccharides conjugated to diphtheria toxoid showed there was a rise in tetanus or diphtheria specific IL-5 respectively following immunisation (373). In adults, however, there was also a significant rise in IFNγ production that was not seen in the toddlers in this study, further emphasising the skewing to Th2 in the 12 month old age group.

3.6.5 Risk of exposure to respiratory infections induced Th2 cytokine responses in toddlers PBMC cultures

There is mixed data about the effect that prior respiratory infections have on the balance of cytokines in young children. In this study it was not apparent that there was any detrimental or enhancing effect when toddlers were thought to be at increased risk of exposure to respiratory infections as a result of social mixing with other children in day care or at home. Mahalingham *et al*-2006 suggested that exposure to respiratory syncytial virus (RSV) increases the skewing towards a Th2 response and even increases the risk of developing allergic diseases. The number and severity of infectious episodes with a particular virus, such as RSV, may be the key factor to the development of a Th1 or Th2 like cytokine environment (407). However, infection with other organisms, such as *Bordetella pertussis*, can skew the immune response toward a definite Th1

phenotype and this has also been seen in response to the primary immunisation with whole cell pertussis vaccine (396).

The data on the effect of nasopharyngeal carriage on the cytokine balance in young children is also varied. In this study, known carriage of pneumococci prior to immunisation did not appear to alter the pattern of cytokines secreted when PBMCs were stimulated *in vitro*. It has previously been shown that nasopharyngeal carriage of *Neisseria meningitidis* induced Th0 responses in teenagers with both IL-5 and IFNγ secreted by activated $CD69^{+}CD4^{+}$ T cells (394). In the case of pneumococcal carriage the cytokines induced by carriage are serotype dependent. In a mouse model of carriage, sepsis and pneumonia the levels of cytokine mRNA expression were compared for different serotypes (408). Following inoculation of serotypes 6B or 14 there was a rise in the levels of IL-12 expression, while carriage or pneumonia caused by these serotypes induced TGFβ expression. IL-10 was induced by nasopharyngeal carriage of serotype 14 while carriage of 6B induced TNFα expression which is inhibited by IL-10. The differences in the patterns of cytokine secretion are thought to be one reason for the differences in the invasiveness of the serotypes (408).

The fact that the different serotype capsules can induce different cytokine secretion patterns has also described during the germinal centre reaction following immunisation of mice and humans with pneumococcal vaccines (189, 195). One important aspect of the cytokine profile induced by the serotype capsule is that cytokines affect the ability of memory B cells to migrate back to lymph nodes. It has been previously described that IFNγ (but not IL-4, IL-1β, IL-6 or TNFα,) is required for the up-regulation of CXCR3 on the surface of memory B cells that express IgG1 as part of a Th1 mediated antibody response (257, 348). Thus the cytokine profile induced following episodes of nasopharyngeal carriage of pneumococci or by immunisation with pneumococcal vaccines may either enhance the B cell response or be detrimental depending on the serotype involved and the maturational status of the immune system.

3.7 Conclusion

The data from this pilot study in 12 month old toddlers have confirmed the appearance of antigen specific plasma cells in the peripheral blood following a single dose of Pnc7-CRM197 vaccine. The *in vitro* stimulation of PBMCs revealed the presence of antigen specific AFC that were non secreting *in vivo* and may be derived from memory B cells or plasmablasts. So the B cell response in 12 month olds appears quite similar to that described in adults to polysaccharide vaccines. The cytokine balance in this age group was still more Th2, though there was IFNγ production. The lymphocyte populations consisted mainly of naïve cells expressing co stimulatory molecules such as CD38, HLA-DR on B cells and CD28, CD38 on T cells.

Two main questions have arisen from this work though and one is whether earlier time points would be better for the detection of vaccine specific plasma cells in peripheral blood? To determine this, a small study in adults was designed to fill in the gaps between day 0-6 and day 10-15 and day 28. The second question was whether it was possible to identify the subsets of B cells involved in the response to Pnc7 immunisation.

Chapter 4: The kinetics of the adult human peripheral blood B cell response to either a single or booster dose of Pnc-7 vaccine.

4.1 Abstract

Young adults received either primary or a booster dose of Pnc7 and PBMCs were isolated for the detection of antigen specific plasma cells. Memory B cells were detected by *in vitro* stimulation of PBMCs with SAC+IL-2. B cells were phenotyped by magnetic cell separation in depletion experiments to identify the subsets responsible for IgG secretion.

A primary dose of Pnc7 induced a significant increase in frequency of diphtheria specific and pneumococcal polysaccharide specific plasma cells and memory B cells in young adults. In response to booster immunisation a peak in IgG, IgA and IgM plasma cell frequency was seen at days 6-7 following immunisation. The IgA response was only significant in response to serotype 4. There was also a rise in the frequency of SAC+IL-2 induced memory B cells at day 6 for serotype 4 and diphtheria toxoid and days 6-15 for serotype-23F. Immunisation did not result in elevated levels of serotype 14 specific IgG-AFC.

PBMCs isolated 6/7 days after immunisation were identified as either $CD20^{+}CD27^{+}sIg^{+}CD38^{+}$ or $CD20^{-}CD27^{+}sIg^{lo/-}CD38^{+}$ and antibody secretion was restricted to the $CD38^{+}$, $CD27^{+}$ or $CD20^{+/-}$ fractions following magnetic cell sorting. Further analysis of plasma cell phenotype showed that more IgG secretion occurred in $CXCR4^{-}$ or $CXCR5^{+}$ fractions than in $CXCR4^{+}$ or $CXCR5^{-}$ fractions. These markers distinguish re-circulating plasma cells ($CXCR5^{+}$) and bone marrow bound plasma cells ($CXCR4^{+}$) from one another and also newly formed plasma cells ($CXCR4^{+}$) from plasma cells ejected from bone marrow niches ($CXCR4^{-}$). It was also possible to show that

diphtheria specific plasma cells were $CD5^-$ and secreted IgG while serotype 4 specific plasma cells were predominantly $CD5^+$ rather than $CD5^-$ and secreted IgA more the IgG.

The antibody response to serotype 4, 14 and 23F polysaccharide was predominantly IgG in response to 23F, a mixture of IgG and IgM to serotype 14 and a combination of IgG, IgA and IgM to serotype 4. Serotype 23F induced the highest IgG response, followed by serotype 14 then 4. The IgA response was greatest in response to serotype 4.

Overall, the Pnc7 booster did not alter the serotype 14 IgG response compared to the primary immunisation, but lowered the IgM and IgA response. There was a slightly earlier IgG response in the booster response to 23F and the response to serotype?? was similar after either primary or booster immunisation. By day 28 after a primary or booster dose there was no significant difference in the IgG concentrations, plasma cell frequency or IgG memory B cell frequency between the primary and booster dose groups.

Conclusions

This data clearly shows that plasma cells appear and disappear during a very small window of the early response to vaccination and that this remains unaltered by primary or booster immunisation. There was some evidence of antibody class switching when post booster antibody was compared to post primary antibody isotype distribution. Also the cells isolated on days 6/7 had the phenotype of either mature plasma cells or plasma blasts, showing that Pnc7 immunisation results in the circulation of at least two antibody secreting cell subsets.

4.2 Introduction

Studies of the immune response against the polysaccharide capsule of the pneumococcus have focused largely on the generation of specific serum antibody following immunisation of infants, the elderly or individuals in groups at high risk of pneumococcal sepsis. The original studies using 23 valent polysaccharide vaccine showed that serum antibody was generated but was often

of low avidity and waned over time, providing some protection against invasive disease in elderly adults (92, 94-97). More recent studies using new glycoconjugate vaccines have been less conclusive about the benefits of immunising adults with these new generation vaccines than has been observed in infants (90, 409, 410).

The limited human data tracking the cellular responses following immunisation includes studies of smallpox, tetanus, diphtheria and pneumococcal polysaccharide vaccines (106-108, 111, 181, 188, 261, 370-372).

These studies demonstrate the appearance of antigen specific plasma cells, spontaneously secreting immunoglobulin, in the peripheral blood of humans and mice, at days 5 to 8 after immunisation (106, 107, 111, 261, 374, 375).

The new pneumococcal glycoconjugate vaccines induce T cell dependent responses as is seen following immunisation with protein antigens, such as tetanus toxoid. The studies mentioned above, utilising T independent and dependent antigens, described peaking plasma cell responses seven days after immunisation irrespective of the antigen suggesting that the early stages of the immune response are not affected by antigen type. Thus differences in the quality of the immune response to glyconjugate versus polysaccharide vaccines may be more to do with the type of B cell recruited during later stages of the immune response. Marginal zone B cells are now known to be the main mediators of anti-polysaccharide responses in humans. These cells are more rapidly activated than conventional follicular B cells (301) because of the strong BCR cross-linking and co-stimulation via complement receptor CD21 (315). Thus polysaccharide antigens preferentially co localise with MZB in the splenic marginal zone under the influence of complement (C3d) opsonisation but it has recently been reported that by conjugating the polysaccharide to a protein carrier this dependency is removed and B cells respond to glycoconjugates under the influence of T cell help (411). Infant MZB have low levels of CD21

expression, inhibiting the MZB response to polysaccharides so by conjugating the polysaccharide with protein this deficiency is overridden by recruitment of T cell help and is the basis behind the enhanced efficacy of the glycoconjugate vaccines in the young age groups (316, 318, 411, 412). In adults however, where both the thymus dependent and independent immune responses are intact in is not certain whether use of glycoconjugates will be of benefit?

In Chapter 3 it was shown that plasma cells could be detected in the ELISpot assay directly *ex vivo* following immunisation of 12 month old toddlers with Pnc7. It was also possible to detect IgG-AFC following *in vitro* stimulation of PBMCs with SAC+IL-2 for 5 days. However, uncertainty regarding the kinetics of plasma cell and memory B cell migration through peripheral blood of toddlers, suggested that sampling should be more frequenct and begin at time points earlier than days 9-11.

Therefore, the studies described in this chapter involved the regular sampling of the peripheral blood of adults during the first 15 days after immunisation enabling characterisation of the plasma cell response to Pnc7 vaccine in relation to the vaccine studies mentioned above.

The *in vitro* stimulation of PBMCs enabled the detection of low frequency, antigen specific AFC derived from circulating memory B cells and precursor plasma cells that persist for some time after antigen exposure. These cells, present in the peripheral blood, are distinct from spontaneously secreting cells and require *in vitro* stimulation via the BCR and co-stimulatory molecules before Ig is secreted (228, 259, 354). In-vitro stimulation with SAC (which cross-links the BCR,) and IL-2 (stimulates proliferation of activated B-cells and T-cells present in PBMC cultures,) provides the signals necessary for the expansion of these antigen specific memory B-cell populations, facilitating their detection by ELISpot (183, 372, 376, 377).

The studies described in this chapter examine the kinetics of peripheral blood B cell populations in more detail and also attempt to identify the phenotypes of the immunoglobulin secreting cells. The effect of administering a single or booster dose of Pnc7 on the B cell and antibody response was also investigated.

4.3 Aims

The primary aim of the studies described in this chapter was to establish the kinetics and frequency of *ex vivo* plasma cells and cultured memory B cells in adult peripheral blood following either a single or booster immunisation with Pnc7.

The secondary aim of this study was to determine the phenotype of the B cells involved at the peak of the *ex vivo*, plasma cell response to the Pnc7 conjugate vaccine.

4.4 Subjects and clinical procedures

Two studies were undertaken in adults to determine B cell responses. The first was a pilot study utilising a booster dose of Pnc7 (Booster study) and the second involved the administration of a single dose of Pnc7 (primary dose study).

4.4.1 The booster dose study

Ten healthy adult volunteers (aged 28 – 44 years, 3 male and 7 female,) received a primary dose of the heptavalent pneumococcal-CRM_{197} conjugate vaccine (Pnc7, Wyeth Vaccines, Pearl River, MA,) by intra-muscular injection in the left deltoid. The 0.5ml dose of the vaccine contained a concentration of polysaccharides of 2.0μg/ml for each of serotypes-4, 9V, 4, 18C, 19F, 23F and 4μg/ml of 6B. Each polysaccharide is conjugated to CRM_{197} (mutant diphtheria toxoid) and adsorbed on aluminium phosphate. Blood was drawn prior to vaccination and again seven days later for measurement of serum antibody.

Six of the 10 original volunteers received a booster dose of Pnc7, 12-17 months after the initial dose. Two of these six adults participated in a detailed study of B cell kinetics during the first 2 weeks after booster immunisation. A 20ml blood sample (2ml clotted for serum and 18ml heparinised for PBMCs,) was collected on days 0, 3, 5, 6, 7, 9, 11, 13, 15. The results from these first two subjects enabled a reduction in the number of blood draws, on days 0, 6, 7, 15 and at 4-6 weeks, from the remaining four volunteers. The blood for PBMC isolation was processed as soon as possible after venepuncture. The plasma samples were stored, in aliquots, at –80°C. Informed consent was obtained from the volunteers and the protocol was approved by the Oxfordshire's Research Ethics Committee (OxREC number C02.005).

4.4.2 The primary immunisation study

A second group of twenty adults was recruited to the study under the same ethical application (OxREC number C02.005,) following submission of a substantial amendment. The adults recruited in this study had no previous history of pneumococcal immunisation or disease and the age range was (aged 23 – 49 years, 8 male and 12 female.) Blood was collected as above, 20ml (2ml for serum and 18ml for PBMCs,) on days 0, 6, 7, 15 and at 4-6 weeks after immunisation.

4.5 Methods

Serum or plasma antibody (IgG, IgM and IgA), to the polysaccharide capsule of serotypes 4, 14 and 23F were measured by ELISA as described in Chapter 2, section 2.10.

PBMCs were isolated (as in chapter 2, section 2.4), from the adult donors at the above the time points prior to and following a booster dose of Pnc7 in the booster study and at the same time points in the primary immunisation study.

For the pilot, booster immunisation study IgG, IgA and IgM plasma cells specific for pneumococcal serotypes 4, 14 and 23F, diphtheria toxoid (dip) and tetanus toxoid (tet) were detected by enzyme linked immunospot assay (ELISpot) either directly ex-vivo (chapter 2,

section 2.5), or following 5 days culture with *Staphylococcus aureus* Cowan strain (SAC) and interleukin (IL-) 2 (chapter 2, section 2.6 and 2.7). Flow cytometry (chapter 2, section 2.8), and AutoMACs™ magnetic cell separation (chapter 2, section 2.9), were used to phenotype the responding cells.

For primary immunisation study blood from ten of the twenty adults was studied in the *ex vivo* IgG plasma cell assay and ten samples were studied using the IgG memory B cell assay. The PBMC isolation, *in vitro* stimulation, Elispot and ELISA assays were as described above and in chapter 2.

4.6 Results

4.6.1 The IgG, IgA and IgM plasma cell response following a booster dose of Pnc7 in adults.

Prior to immunisation there was evidence of plasma cell Ig secretion in the peripheral blood of the adults volunteers (median counts of between 5 to 7 IgG-AFC/10^6 PBCMs). There were also low levels of peripheral blood IgM and IgA plasma cells specific for tetanus and diphtheria toxoid, serotype 4, 14 and 23F (fig.4.4a-e). Median frequencies of 0 to 3 IgM or IgA-AFC/10^6 PBMCs equated to between 33%-66% of individuals with pre-existing, antigen specific plasma cells.

The data shown in fig.4.1a-e, show detailed time course of IgG secreting plasma cell frequency in two adult volunteers. Following booster immunisation the plasma cells appeared in peripheral blood by day 5, peaking in frequency by days 6-7 and a returning to baseline by day 15. When studied in all six individuals it was clear that this pattern was consistent in timing, with IgG-AFC frequencies rising significantly above baseline for all of the vaccine related antigens at day 6-7 (fig.4.2 b-e). The magnitude of the response was variable between individuals and for each

antigen (fig. 4.2 a-e). There were no detectable alterations in the frequency of tetanus toxoid specific plasma cells, except for 2 individuals, in whom there was a rise at day 6-7 (fig.4.1a and 4.2a) though this was not significant.

Following booster immunisation the frequency of IgM plasma cells specific for diphtheria toxoid (the carrier related antigen) increased from 3 to 8 IgM-AFC/10^6 PBMCs and the percentage responders was 80% at day 15 but this response was not significant (fig.4.4.b). There was a non significant elevation in frequency of IgM plasma cells specific for the capsular polysaccharides of serotypes-4, 14 or 23F on day 6, with a return to baseline levels by day 15 (fig.4.4c-e). The post booster IgA plasma cell response to serotype 4 (fig.4.4c), rose significantly 6-7 days after immunisation, peaking at 256 IgA-AFC/10^6 PBMCs, but waning again by day 15.

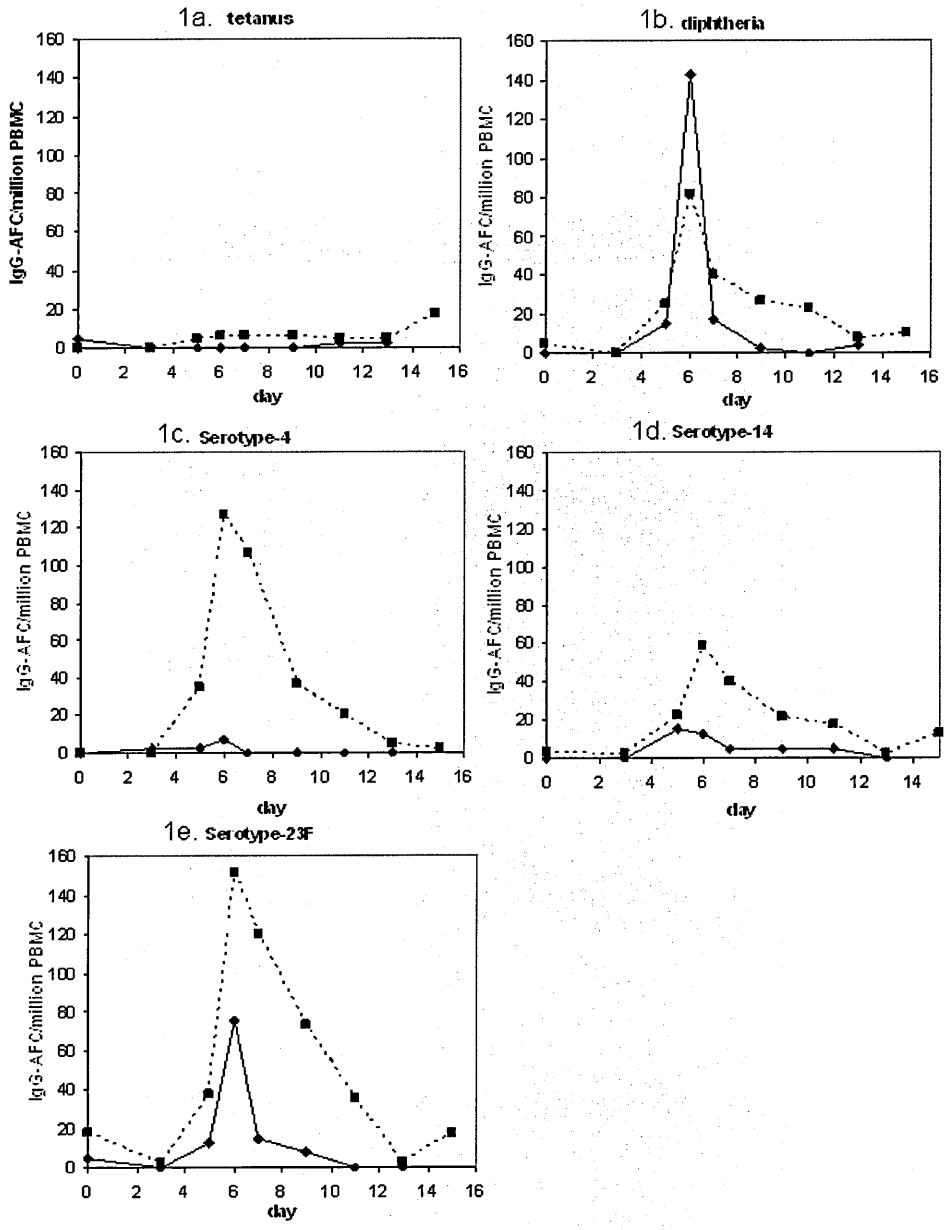


Figure 4.1 The frequency of spontaneously secreting, IgG-AFC in the peripheral blood of two adult volunteers before and at various time points from 3 to 15 days post immunisation.
The IgG-AFC specific for tetanus toxoid (1a), diphtheria toxoid (1b), serotype 4 (1c), serotype 14 (1d) and serotype 23F (1e), were enumerated by ELISpot. The data are expressed as the number of antigen specific IgG-AFC / 10^6 PBMCs.

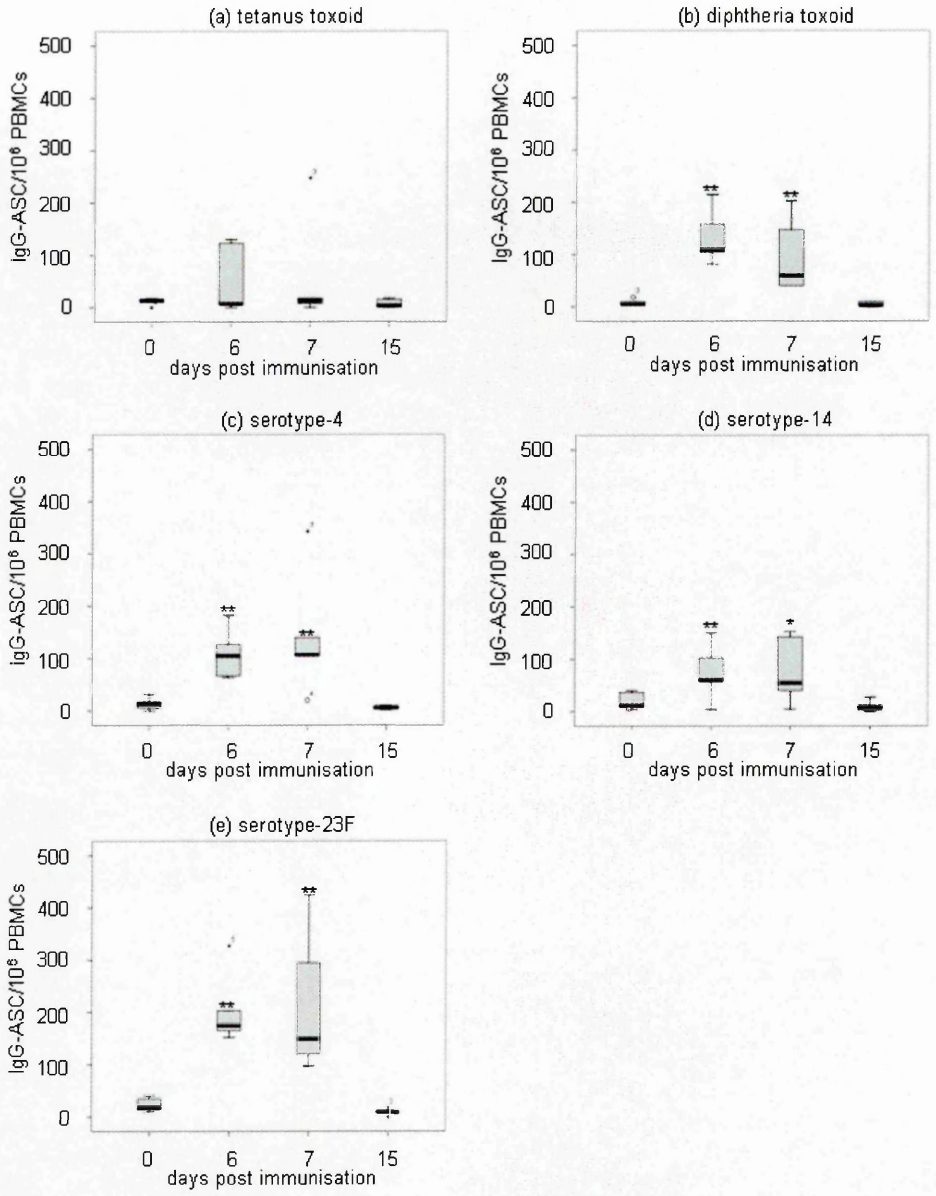


Figure 4.2 The post Pnc7 booster frequency of IgG secreting plasma cells isolated from the peripheral blood of 6 adults on day 0 and on days 6, 7, 15.
IgG-AFC specific for tetanus toxoid (a), diphtheria toxoid (b), serotype 4 (c), serotype 14 (d), and serotype 23F (e), were enumerated by ELISpot. The data are expressed as the number of antigen specific IgG-AFC/10^6 PBMCs. (**p=<0.05, *p= ≤0.08). The box represents the 25^{th} and 75^{th} quartiles and the whiskers are the outliers.

4.6.2 The IgG, IgA and IgM memory B cell response to a booster dose of Pnc7 in adults.

Prior to booster immunisation (day 0), *in vitro* stimulation of PBMCs with SAC+IL-2 revealed a low but detectable frequencies of memory B cell derived IgG-AFC specific for the three pneumococcal serotypes in the ELISpot assay with numbers of IgG-AFC/10^6 cultures PBMCs for serotype 23F>14>4 in frequency (fig. 4.3d-e). Diphtheria specific memory IgG-AFC induced by SAC+IL-2 were not detectable at day 0 (fig.4.3b).

There was evidence of a strong baseline level of memory B cell derived IgM-AFC specific for tetanus, diphtheria, serotype 4, 14 and 23F, while IgA memory was at the limits of detection for these assays (fig.4.4f-j).

Following administration of a booster dose of Pnc7 there was a non-significant rise in tetanus toxoid specific memory B cell derived IgG-AFC (fig.4.3a). Diphtheria specific IgG-AFC induced by SAC+IL-2 rose significantly, peaking at day 6, for all but one individual, where the rise in the frequency was delayed until day 15. Diphtheria specific IgG-AFC declined after day 15 but remained detectable even at days 28-42, with the exception of one individual (fig.4.3b). In response to the pneumococcal capsular polysaccharides there was a more gradual rise in the memory B cell IgG-AFC response than was seen in the plasma cell response. In response to serotype 14 there was an equal number of responders and non-responders by day 6 following the booster dose of Pnc7. This response returned to baseline by day 15 for all except two of the vaccinees (fig.4.3d). Most individuals responded with an increase in frequency of serotype 4 and 23F specific IgG-AFC (fig.4.3c+e). The response of one individual to serotype 23F was of far greater magnitude than any of the other individuals, and coincided with also achieving the highest frequency of diphtheria specific IgG-AFC (fig.4.3b+e). The same individual responded less well to serotype 4 and 14.

The memory IgM-AFC response induced by booster immunisation was elevated in response to the vaccine related antigens and also to tetanus toxoid by day 6 (fig.4.4f-j). The magnitude of IgM memory cell frequency, following SAC+IL-2 stimulation of PBMCs, was greater even than the IgG responses seen in fig.4.3. However the increases in IgM memory B cell frequency were not significantly elevated above baseline at any point. The IgA memory cell frequency was also elevated by day 6, most notably for serotype-4 where IgA cell frequency was elevated at day 6($p=0.08$) and reached significance by day 15 ($p=<0.05$, fig.4h). The magnitude of the IgA memory response was lower than the IgM memory response and also the IgG memory response except for serotype 4 which induced a higher frequency of IgA secreting plasma cells and *in vitro* induced IgA-AFC than either serotype 14 or 23F.

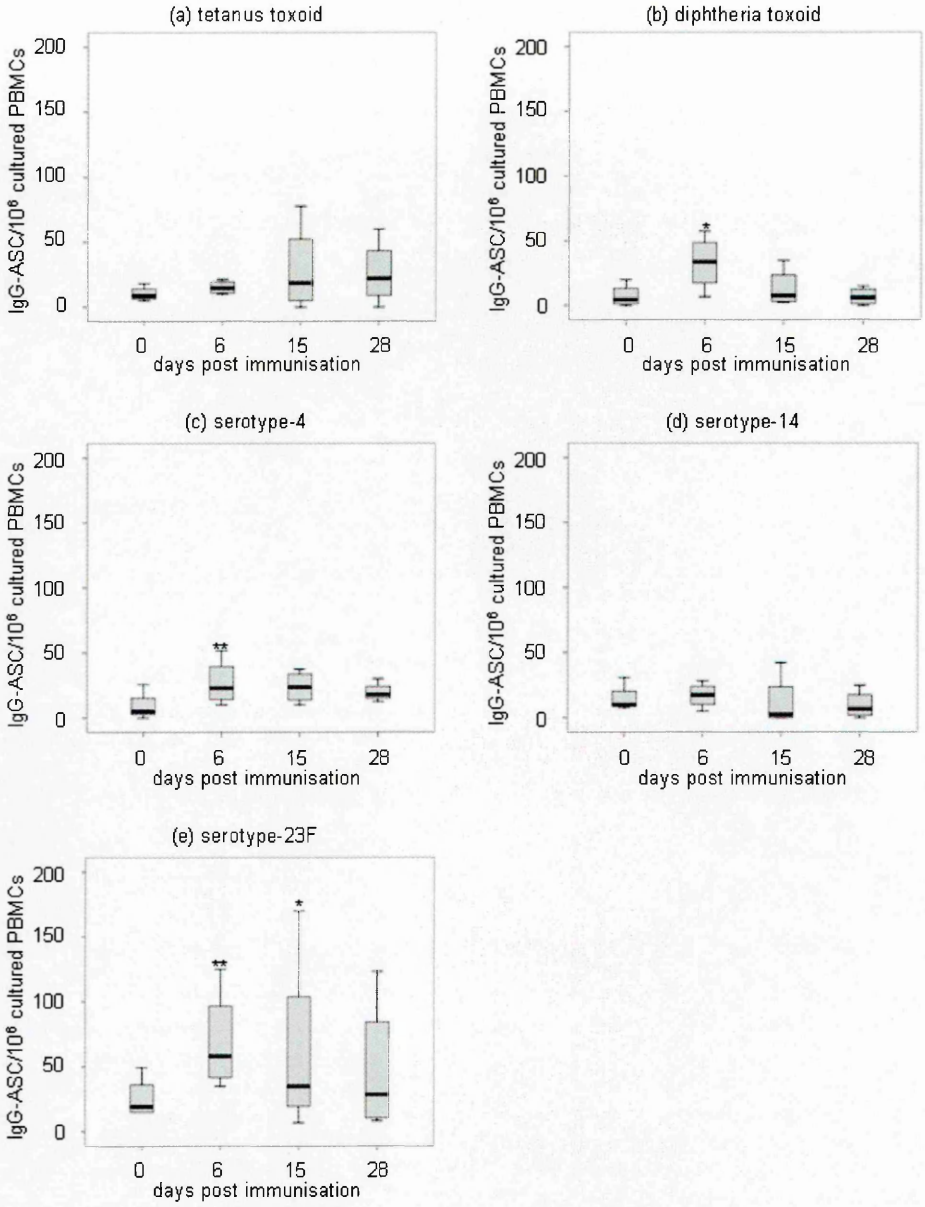


Figure 4.3 The post Pnc7 booster frequency of IgG memory B cells in the peripheral blood of 6 adults on day 0 and on days 6, 7, 15.

Following *in vitro* stimulation of PBMCs with SAC+IL-2 the IgG-AFC frequency in response to tetanus toxoid (a), diphtheria toxoid (b), serotype 4 (c), serotype 14 (d), and serotype 23F (e) was enumerated by ELISpot. The data are expressed as the number of antigen specific IgG-AFC/10^6 PBMCs. The box represents the 25^{th} and 75^{th} quartiles and the whiskers are the outliers. (**p=<0.05, *p= ≤0.08).

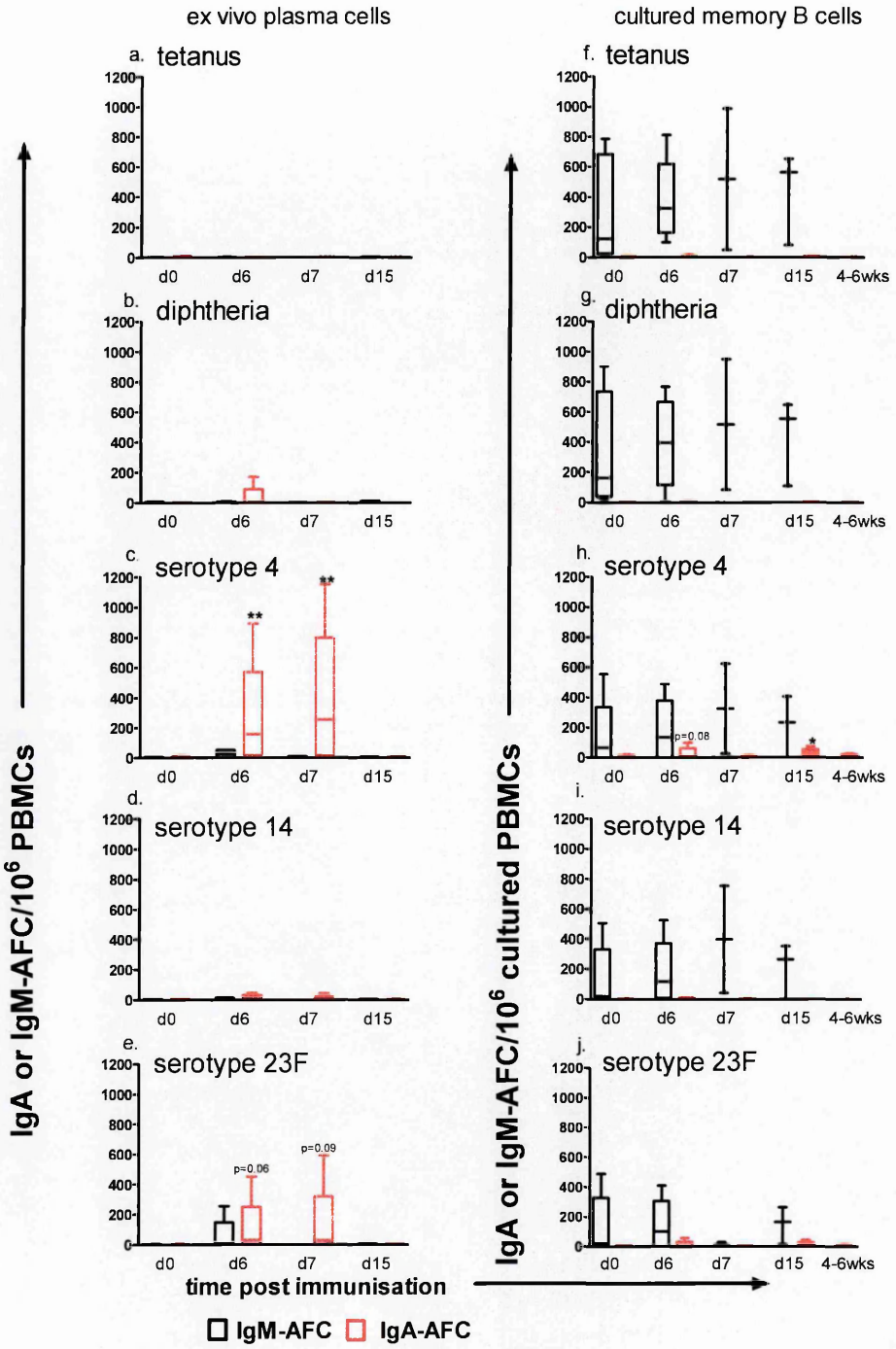


Figure 4.4 The post Pnc7 booster frequency of spontaneously secreting and memory B cell derived IgM and IgA-AFC in peripheral blood of adults.
PBMCs were isolated on days 0, 6,7, 15 and at 4-6 weeks post immunisation for the detection of IgM (black) and IgA (red) plasma cells by ELISpot (a-e), or were stimulated for 5 days with SAC+IL-2 before being incubated on antigen coated Elispot plates for the detection IgM and IgA memory B cell responses (f-j). Data are expressed as IgM or IgA-AFC/10^6 PBMCs or /10^6 cultured PBMCs. There were between 2 and 6 individuals at each time point.

4.6.3 The characteristics of IgG secreting cells isolated 7 days after booster dose of Pnc7 in adults.

PBMCs isolated on the peak day of the plasma cell response (day 7) to a booster dose of Pnc7, were separated by AutoMACs™ on the basis of, CD20, CD27 or CD38 expression. The frequencies of plasma cells, specific for tetanus (tet) and diphtheria (dip) toxoid and serotype 4, 14, and 23F polysaccharides were quantified within each fraction and in the un-separated PBMC sample (fig.4.5a). Plasma cells specific for serotype 4, 14, 23F and diphtheria toxoid were present in both the $CD20^+$ and $CD20^-$ fractions of the PBMC population, although they appeared to be more frequent in the $CD20^+$ fraction. Tetanus toxoid specific plasma cells were present in the un-separated PBMC sample, but were undetectable after magnetic cell sorting. There was almost a complete absence of plasma cells within the $CD27^-$ and $CD38^-$ fractions with all immunoglobulin secreting activity appearing within the $CD27^+$ and $CD38^+$ fractions.

The phenotype of these magnetically separated immunoglobulin secreting cells was investigated further by flow cytometry. Cell surface immunoglobulin expression was compared between the $CD27^+$ secreting fraction and the $CD27^-$ non-secreting fraction (fig.4.5b). The percentage of IgG, IgM and IgD expressing cells was higher in the non-secreting, $CD27^-$ fraction than in the $CD27^+$ fraction.

Further phenotypic analysis was carried out on the CD20, and CD38 separated fractions. The presence or absence of both CD27 and surface immunoglobulin isotypes was assessed in the CD20 ($^+$ and $^-$) and CD38 ($^+$ and $^-$) fractions (fig.4.5c). The $CD27^-$ B-cells in all of the fractions expressed far more surface Ig than did the memory, $CD27^+$ B cells. The $CD20^+CD27^+$ B-cells had higher expression of Ig than any of the other $CD27^+$ populations. Overall, the fractions with antibody secreting activity in the ELISpot assay ($CD27^+CD20^{+/-}$ or $CD27^+CD38^+$), had lower surface expression of Ig than the non-secreting fractions.

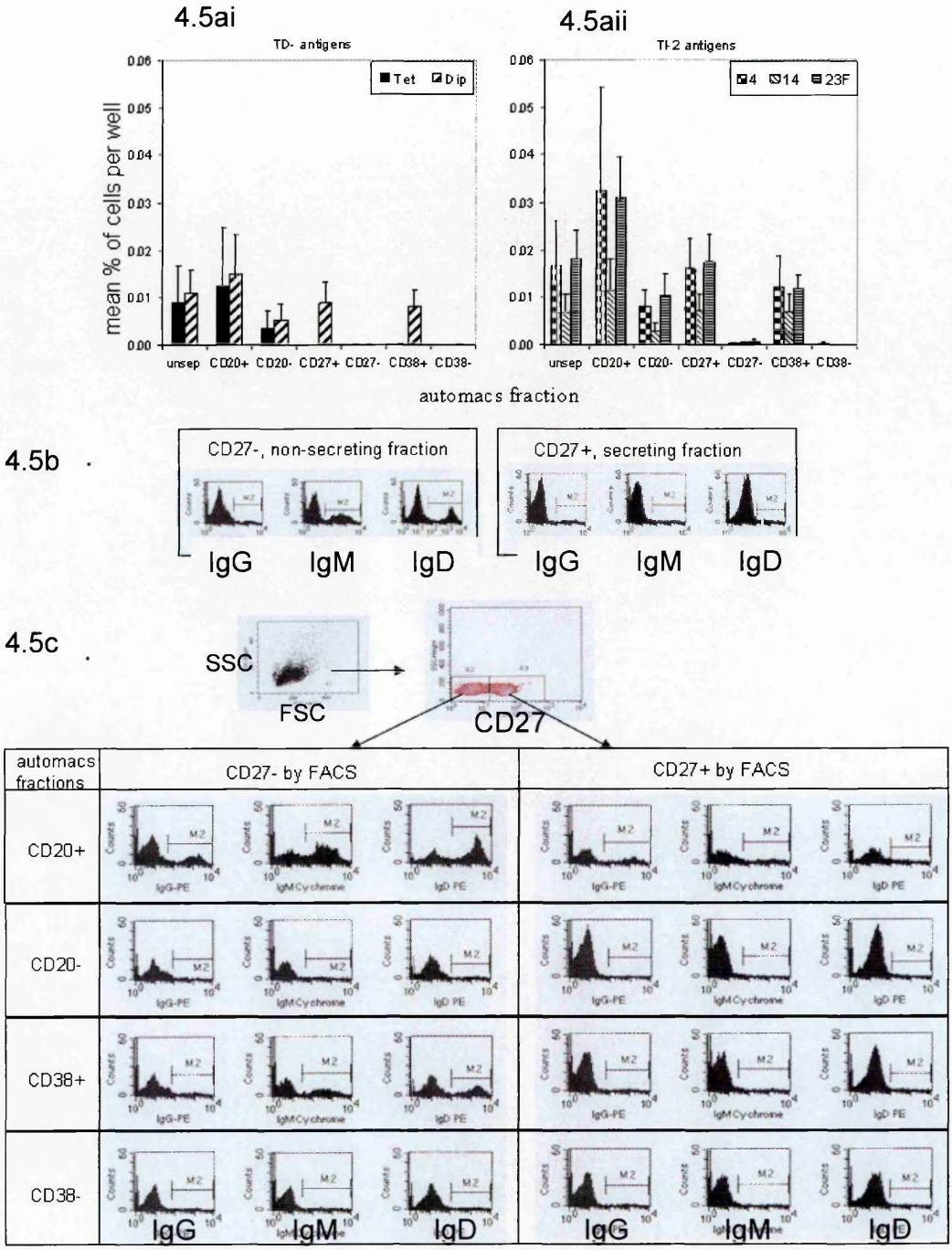


Figure 4.5 The phenotype of IgG plasma cells isolated on day 7 after a booster dose of Pnc7 in adults. The percentage of antigen specific IgG plasma cells detected in each magnetically sorted fraction is shown in (4.5a). The data represents the mean and standard error from 3 different donors and is expressed as the percentage of the total cells per well. Tetanus (tet) and diphtheria (dip) toxoid specific IgG-AFC are shown in (4.5ai) and polysaccharides 4, 14 and 23F IgG-AFC in (4.5aii). The expression of IgG, IgM and IgD in the $CD27^+$ and $CD27^-$ fractions is shown in 4.5b. The expression of IgG, IgM and IgD with and with CD27 expression is shown for the $CD20^{+/-}$ and $CD38^{+/-}$ fraction in 4.5c. The data in 4.5b+c are representative data from one experiment of three.

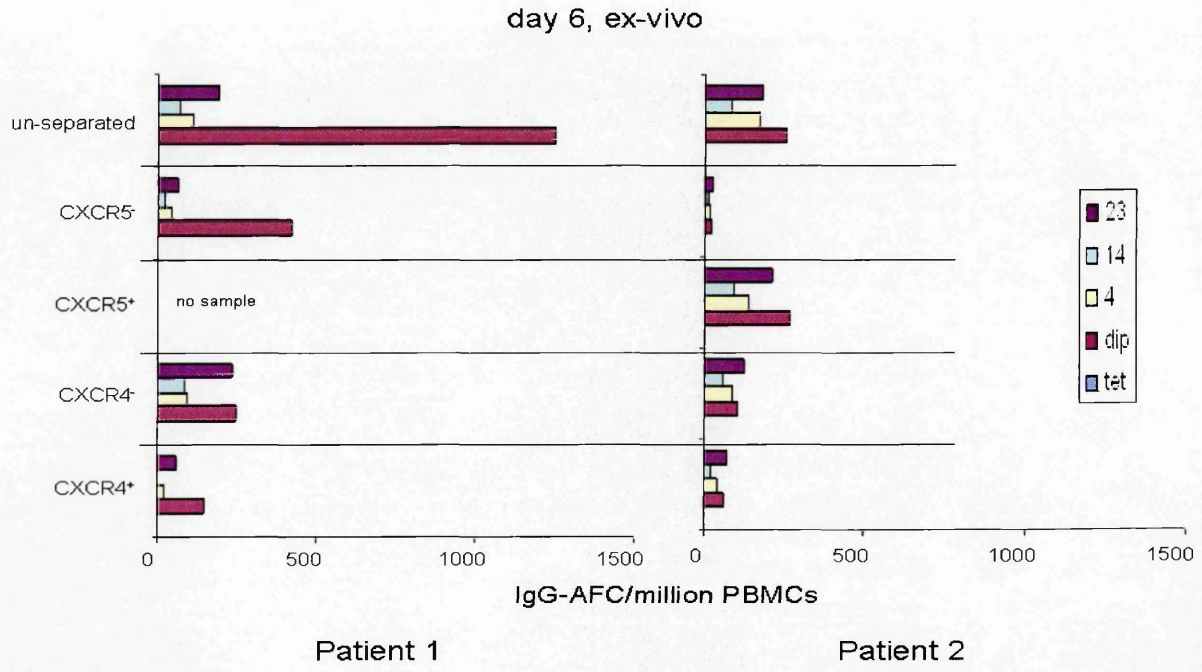


Figure 4.6 Separation of PBMCs on the basis of the chemokine homing receptors CXCR4 or CXCR5 expression.
PBMCs were isolated from two adult donors on day 6 following a primary dose of Pnc7. The PBMCs were separated on the basis of CXCR4 or CXCR5 surface expression. These fractions, along with unseparated PBMCs were seeded on to antigen coated ELISpot wells for the detection of spontaneously secreting IgG-AFC in peripheral blood. IgG-AFC specific for tetanus and diphtheria toxoid and the capsular polysaccharides from pneumococcal serotypes 4, 14 and 23F were detected and the data are expressed as the number of IgG-AFC/10^6 PBMCs.

4.6.4 Depletion of CXCR4$^+$ or CXCR5$^+$ IgG secreting cells isolated 6 days after booster dose of Pnc7 in adults

On day 6 after immunisation PBMCs from two study subjects were separated by magnetic cell sorting on the basis of either CXCR4 or CXCR5 expression. The separated fractions were then re-suspended and seeded into ELISPOT plates and allowed to spontaneously secrete antibody overnight. The CXCR4- and CXCR5+ fractions contained a greater proportion of the antibody

secreting activity (fig.4.6). There were IgG secreting plasma cells with specificity for diphtheria toxoid and the three pneumococcal polysaccharides (4, 14 and 23F) in all fractions. However, there were no IgG secreting plasma cells present in the sorted fractions that were specific for tetanus toxoid.

4.6.5 IgG secretion by cells sorted on the basis of CD5 expression 6 days after a booster dose of Pnc7 in adults.

PBMCs from obtained from an adult donor, 6 days after booster Pnc7 immunisation, were depleted of $CD3^+$ T cells and then separated by AutoMACs™ magnetic cell separation on the basis of CD5 expression (fig.4.7). Within each fraction ($CD5^+$, $CD5^-$ and unseparated PBMCs) the percentage of the $CD19^+$, $CD3^+$, $CD19^+CD5^+$, $CD27^+$ and $CD27^+$ were determined by flow cytometry (fig.4.7a). The $CD19^+$ B cell population was enriched in both the $CD5^+$ (11% $CD19^+$) and $CD5^-$ (18% $CD19^+$) fractions compared to unseparated PBMCs (5% $CD19^+$). The $CD5^-$ fraction was totally depleted of $CD3^+$ T cells while 3% $CD3^+$ T cells remained in the $CD5^+$ fraction after depletion compared to unseparated PBMCs (70% CD3+ T cells). The percentage of $CD19^+CD5^+$ B cells was enriched in the $CD5^+$ fraction (4%) while there was less than 1% in unseparated PBMCs and non in the $CD5^-$ fraction. The percentage of $CD27^+$ B cell was less than 10% in both the $CD5^+$ and $CD5^-$ fractions while there were 40% $CD27^+$ lymphocytes in the unseparated fraction. The proportion of the fractions that were $CD27^-$ was similar, approximately 35-41%.

The secretion of IgG and IgA within the $CD5^+$ and $CD5^-$ fractions and the unseparated PBMCs was quantified by antigen specific ELISpot. The number of IgG and IgA-AFC per/10^6 sorted cells was calculated and is presented in figure 4.7b. In the unseparated PBMCs no tetanus or serotype 4 specific IgG-AFC were detected but there were diphtheria specific IgG-AFC. IgG-AFC with specificity to all three antigens (tet, dip and serotype 4), were enriched in the $CD5^-$ fraction. The serotype 4 response was enriched to a greater extent in the $CD5^+$ fraction but this

was not so for the diphtheria and tetanus IgG response. More evident was that while no IgA response was detected in response to tetanus and diphtheria, there was a 7-fold increase in enrichment of IgA-AFC specific for serotype 4 in the CD5+ fraction compared to the unseparated fraction. Therefore the response to the polysaccharide capsule of serotype 4 was mediated by IgA-AFC to a greater extent than IgG and by $CD5^+$ B cells than by $CD5^-$ B cells. From the phenotyping data in section 4.5.3 it can be assumed that the IgG-AFC are all $CD27^+$ as there was no IgG secretion in the $CD27^-$ fraction.

4.6.6 The serotype specific IgG, IgA and IgM antibody response following a booster dose of Pnc7 in adults.

Adults received a booster dose of Pnc7 and the IgG, IgA and IgM serum anti-capsular antibody concentrations, against pneumococcal serotypes 4, 14 and 23F were quantified prior to immunisation (day 0) and on days 6, 7, 15 and 36-42 after immunisation (fig.4.8a-c). The GMC of IgG prior to administration of the booster dose (12-17 months after a primary dose), was above the protective threshold of 0.20μg/ml(362) for serotypes 4, 14 and 23F (0.62μg/ml, 14.78μg/ml and 4.51μg/ml respectively). Following the booster immunisation there was a rapid and steady rise in IgG, IgA and IgM levels from day 6, peaking at day 15 and remaining elevated at days 36-42 for serotypes 4, 14 and 23F. Serotype 4 polysaccharide induced only a minimal rise in antibody peaking significantly above baseline by day 15 for IgA ($p=<0.05$), and IgM ($p=<0.01$). Serotype 14 polysaccharide induced no significant rises in antibody, but had the highest pre-booster levels of IgG and IgM. Serotype-23F induced significant levels of IgG ($p=<0.05$), IgM ($p=<0.001$), and IgA ($p=<0.01$), by day 15 post booster. The elevated levels of IgG, IgA and IgM antibodies were still maintained by days 36-42 for all 3 serotypes (fig.4.8a-c).

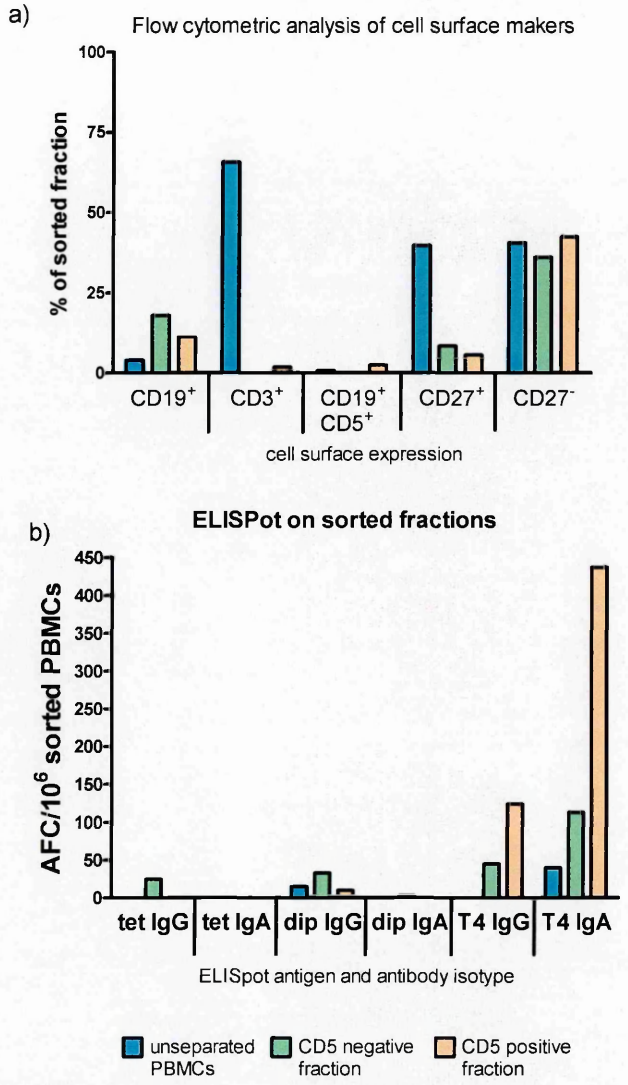


Figure 4.7 The phenotype and IgG or IgA-AFC frequency of PBMCs separated on the basis of CD5 expression.

PBMCs isolated 6 days after a booster dose of Pnc7 were separated by magnetic cell separation based on the surface expression of CD5. T cells were depleted prior to CD5 separation by the use of anti-CD3 magnetic beads and then the CD3 negative fraction was then separated into $CD5^+$ (orange) and $CD5^-$ (green) fractions. Unseparated PBMCs (blue) were retained as the control for surface marker expression and immunoglobulin secretion in the original sample.

4.7a) Purity and enrichment of the separated fractions was analysed by flow cytometry on the basis of $CD19^+$, $CD3^+$ $CD19^+CD5^+$, and $CD27^{+/-}$ expression and the data represent the percentage of each fraction expressing the marker. b) The remaining cells from each fraction were seeded onto antigen coated ELISpot plates for the detection of IgG or IgA secreting cells specific for tetanus toxoid, diphtheria toxoid or pneumococcal serotype 4 polysaccharide. The ELISpot data is expressed as the mean number of IgG or IgA-AFC/10^6 PBMCs isolated on day 6 after immunisation.

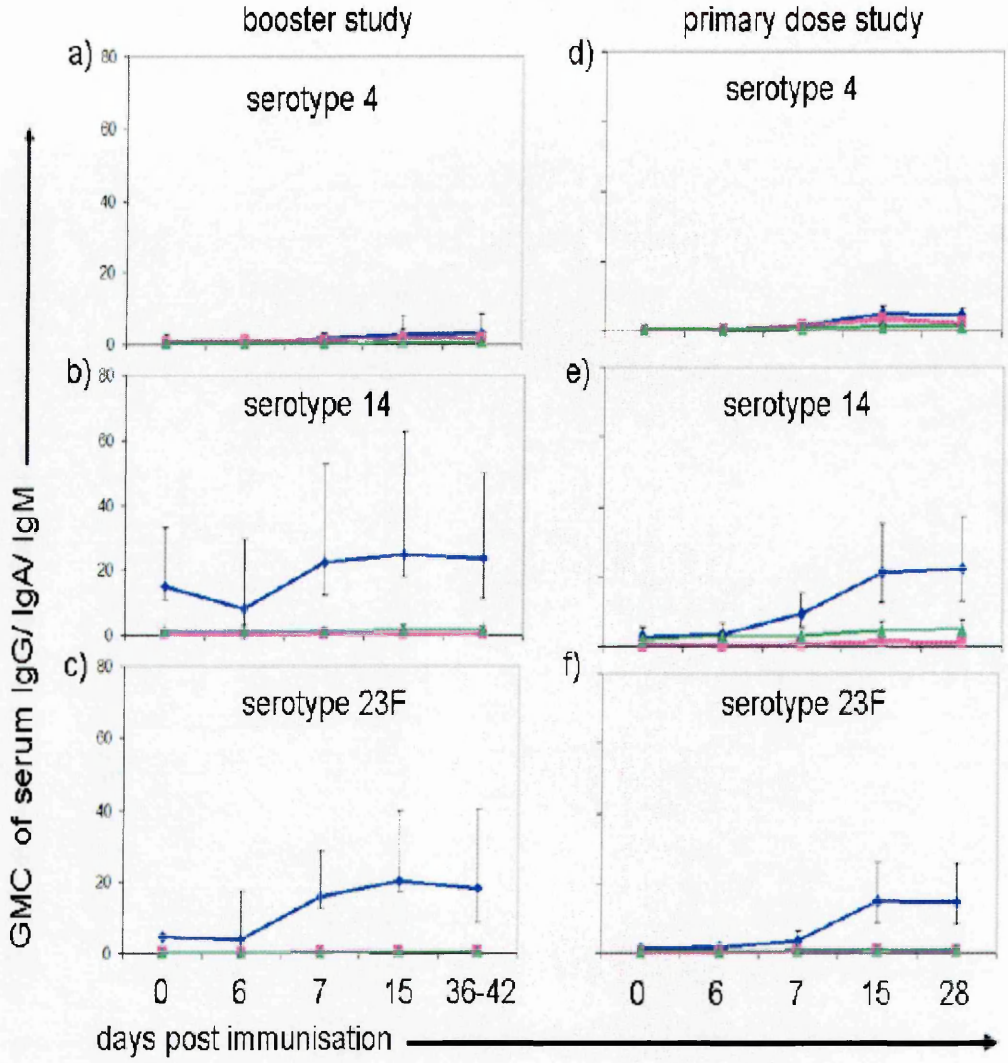


Figure 4.8 Pneumococcal serotype specific antibody in serum of adults before and after a booster (figures a-c) or primary dose (figures d-f) of Pnc7 vaccine.
Six adults received a booster dose of Pnc7 12 to 17 months after receiving a first dose of the same vaccine (a-c). A second group of twenty adult volunteers received a primary dose of Pnc7 (d-f). Blood was drawn on day 0 and then days 6, 7, 15 36-42 days after the booster dose or upto 28 days after the primary dose. IgG (♦) IgA (■) and IgM (▲) were measured against serotypes 4 (a+d) 14 (b+e) and 23F (c+f). Data represent the GMC with 95% confidence intervals.

4.6.7 The serotype specific IgG, IgA and IgM antibody response following a primary dose of Pnc7 in adults.

A group of twenty adults were immunised with a single dose of Pnc7 and the serum antibody levels were quantified by ELISA. The kinetics of the antibody response to each of the serotypes (4, 14 and 23F), following the single dose of Pnc7 (fig.4.8d-f) were similar to those seen following the booster dose of Pnc7 (fig 4.8a-c). All three serotypes induced significant increases in IgG, IgA and IgM by day 6 and the concentrations continued to increase until day 15 where they then remained level until day 28.

Serotype 4 induced a small and mixed response of IgG/IgA/IgM (fig.4.8c), with significant rises in all isotypes by days 6 and 7 ($p=<0.0001$) post immunisation that remained above baseline even by day 28 ($p=<0.0001$). Antibodies directed against serotype 14 (fig.4.8e) were higher at baseline (IgG=1.92μg/ml, IgA=0.19μg/ml and IgM=2.31μg/ml), than those against serotype 4 (IgG=0.34μg/ml, IgA=0.11μg/ml and IgM=0.26μg/ml), and 23F (IgG=1.12μg/ml, IgA=0.07μg/ml and IgM=0.50μg/ml). The levels of IgG, and IgM remained higher against serotype 14 than those against serotypes 4 and 23F throughout the time course. From day 6 onwards serotype 4 induced more specific IgA than seen in response to serotypes 14 and 23F, while serotype 14 induced the most IgM.

4.6.8 Comparison of the serum IgG, IgA and IgM antibody responses after either, a primary or booster dose of Pnc7 in adults.

At baseline (d0) there were some differences in antibody levels between the primary dose and booster group (fig.4.9a-j). For instance there were significantly higher pre-immunisation levels of IgG specific for serotype 4 and 14 in the booster group (fig.4.9a-c). The pre-immunisation levels of IgA (d0, fig.4.9d-f), were significantly higher for serotypes 4 and 23F in the booster group. There were no differences in pre-immunisation levels of IgM between the groups.

Following immunisation there were no significant differences in the serum IgG response to any of the serotypes (4, 14 and 23F), whether a primary or booster dose was given (fig.4.9a-c). There was a trend for more rapid and higher IgG production in response to serotypes 14 and 23F in the booster group compared to the primary group but this was not significant. There was a trend for more IgA in the primary dose group and this was significantly higher than in the booster group for serotype 14 at day 15. The IgA response to serotype 4 was initially higher in the booster group but by day 15 after immunisation this was reversed and IgA remained higher in the primary dose group, though this was not significant (fig.4.9d). The IgM response was greatest for serotype 14 and was higher in the primary dose group compared to the booster dose group, significantly so at days 15 and 28.

Overall, figure 4.9 shows that the same isotype to serotype relationship was seen following either a primary or booster dose of Pnc7. In the IgG responses serotype 14>23F>4. In fig 4.9d-f, it can be seen that the IgA response was serotype 4> 14≥ 23F and the pattern for the IgM response was serotype 14>4>23F (Fig 4.9g-i).

4.6.9 The IgG plasma cell response in the peripheral blood of adults following either a primary or booster dose of Pnc7.

The kinetics of the IgG plasma cell response in adults were the same following either a primary or booster dose of Pnc7 vaccine, with peak plasma cell frequencies at days 6 to 7 (fig4.10a-e). The magnitude of the diphtheria specific response (fig.4.10b), was significantly lower in the booster group, than in the primary dose group, suggesting that the CRM197 carrier response was not boosted by re-immunisation. There were no differences in the response to the capsular polysaccharides of serotypes 4, 14 and 23F.

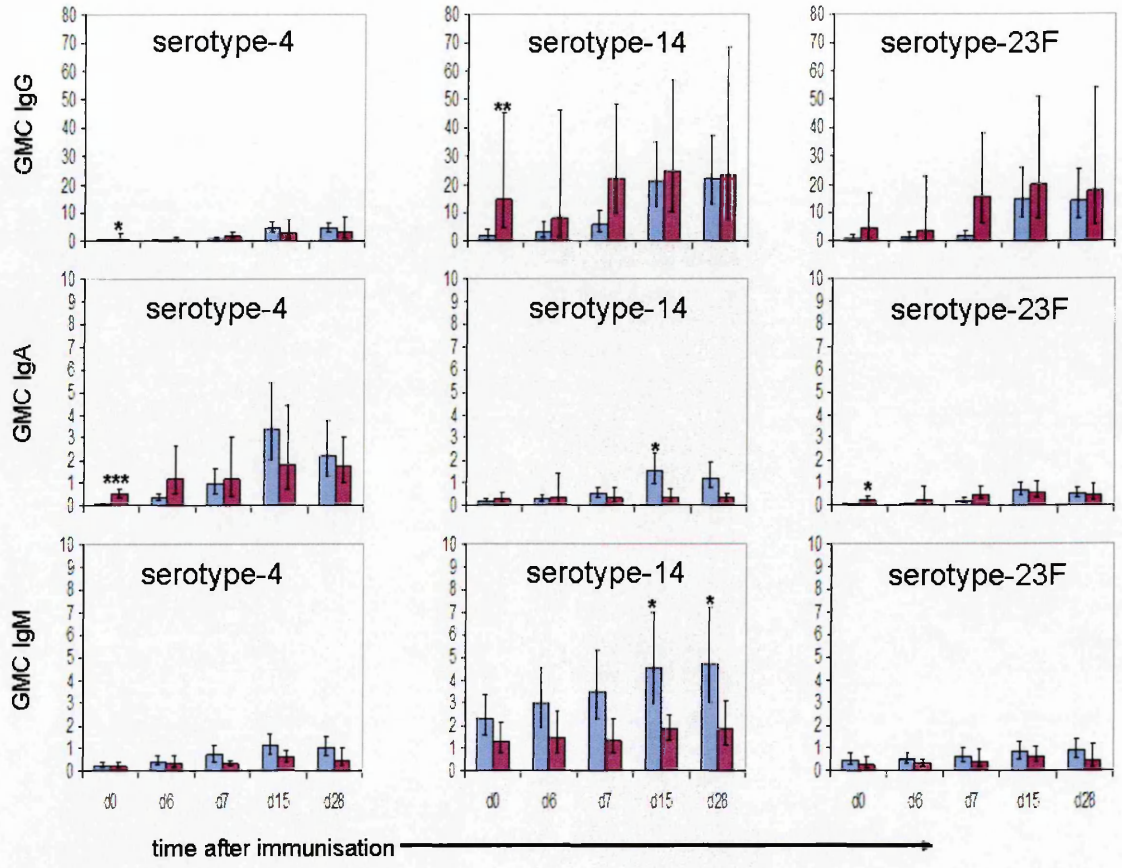


Figure 4.9 Comparison of anti-capsular antibody isotype and kinetics in serum of adults following a primary or booster dose of Pnc7.
Serum was collected at each of the time points prior to and following immunisation of adults receiving either a primary (blue) or booster dose (red) of Pnc7. IgG, IgA and IgM anti-capsular polysaccharide antibodies against serotypes 4, 14 and 23F were quantified by ELISA and compared between the groups. The data are expressed as the geometric mean concentration of antibody with 95% confidence intervals. The significance levels were calculated using un-paired ttest function on Prism V4.

4.6.10 The peripheral blood memory B cell response following either a primary or booster dose of Pnc7 in adults.

Following *in vitro* stimulation of PBMCs with SAC+IL-2 at each time point the frequencies of memory B cell derived IgG-AFC were compared between the booster group and the primary dose group. There was no difference in tetanus toxoid specific memory B cell frequency between the groups at any of the time points (Fig 4.11a). There was a trend for higher frequencies of

diphtheria specific memory B-cells in the primary dose group than in the booster group (fig.4.11b), but this difference was not apparent prior to immunisation (d0) and was not significant. The frequencies of capsular polysaccharide specific memory B cells was higher were similar between the groups except in response to serotype-23F at day 6 post immunisation, where the booster group had a higher frequency of IgG-AFC ($p=0.008$, fig.4.11e).

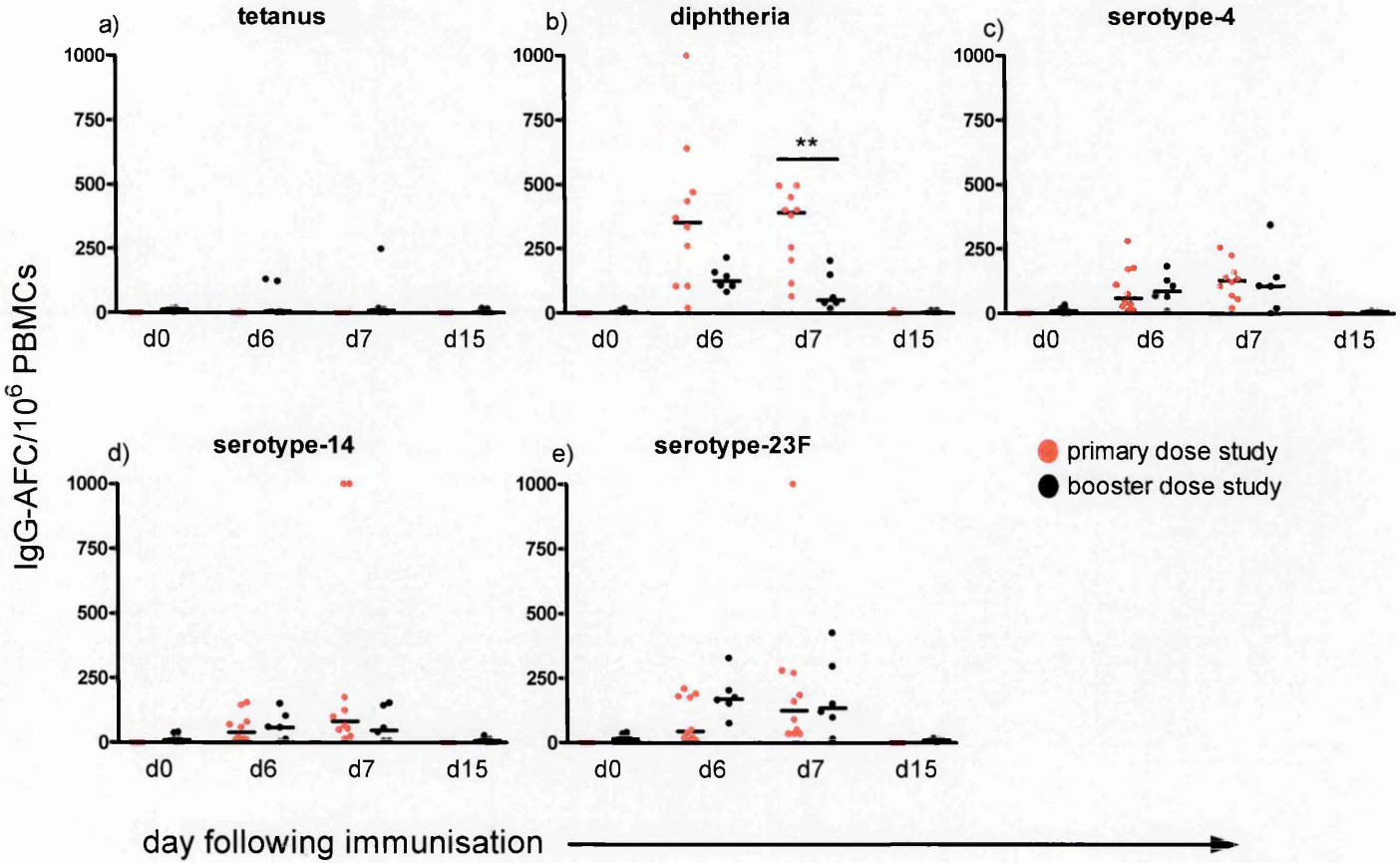


Figure 4.10 The frequency of spontaneously secreting IgG-AFC in the peripheral blood of adults following a primary or booster dose of Pnc7. PBMCs were isolated at each time point following immunisation and incubated on antigen coated ELISpot plates. Spontaneously secreting IgG-AFC specific for tetanus, diphtheria and serotypes-4, 14 and 23F polysaccharides were quantified. The data represent the number of individuals receiving either a primary (red) or a booster dose (black), with the bar representing the median spot count / 10^6 PBMCs.

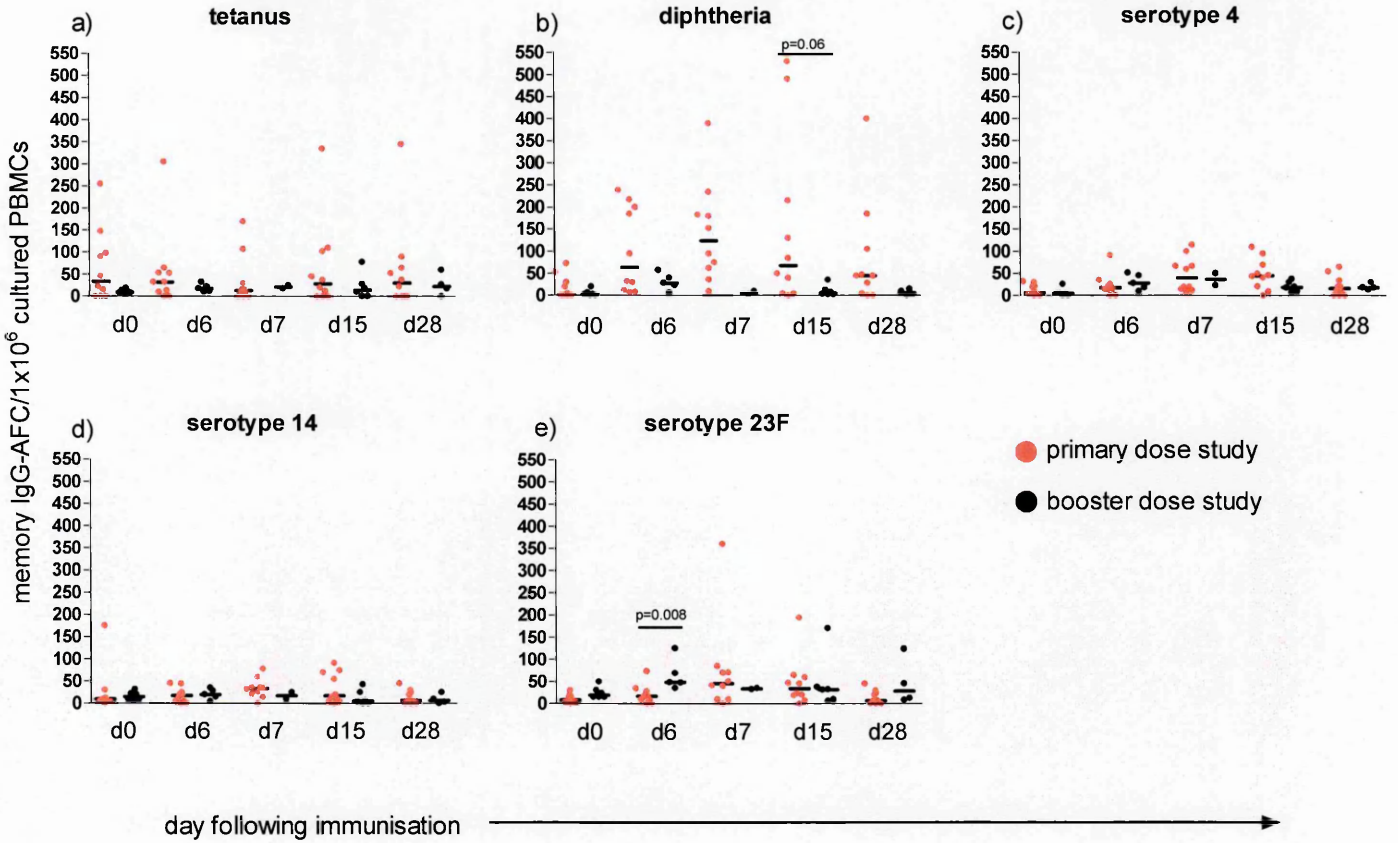


Figure 4.11 The frequency of memory B cell derived IgG-AFC in the peripheral blood of adults after a primary or booster dose of Pnc7.
PBMCs were isolated at each time point and stimulated with SAC+IL-2 for 5 days. Following this the cells were harvested and incubated on antigen coated ELISpot plates for the detection of IgG-AFC specific for tetanus, diphtheria and serotypes-4, 14 and 23F polysaccharides. The data represent the number of individuals receiving a primary (red) or a booster dose (black) of Pnc7 and the bar represents the median IgG-AFC / 10^6 cultured PBMCs.

4.7 Discussion

In this study the peripheral blood kinetics and phenotype of populations of B cells appearing after immunisation of adults with the glycoconjugate vaccine, Pnc7 have been described. Plasma cells that spontaneously secrete antibody *ex vivo* appear briefly in peripheral blood, peaking on days 6-7 following immunisation, and are of two phenotypes ($CD20^+CD38^+CD27^+$ or $CD20^-CD38^+CD27^+$). Both of these subsets have low surface immunoglobulin (Ig) expression. Furthermore, the appearance of these antibody secreting cells is accompanied by a more sustained, though lower magnitude, rise in appearance of a non-Ig secreting B-cell subset in the peripheral blood that have the *in vitro* characteristics of memory B cells or plasmablasts.

4.7.1 Pnc7 immunisation induced elevated frequencies of vaccine specific plasma cells in adult peripheral blood.

This study has identified a population of spontaneous Ig-secreting cells that produce antibody to the capsular polysaccharides of the Pnc7 vaccine. The cells appeared by day 5 and peaked at days 6-7 following booster immunisation, but had fallen to undetectable levels by day 15. There was a simultaneous rise in serum anti-capsular IgG, IgA and IgM antibodies from day 6 following the administration of the booster dose with a peak by day 15.

Similar kinetics have been described following immunisation of toddlers and adults with Pnc7 or 23-valent pneumococcal polysaccharide vaccine (107, 111, 112, 261) and in mice (375).

In adult studies from the early 1980's by Kehrl *et al* (108-110) the kinetics of the B cell response to a single dose of 14 valent pneumococcal polysaccharide vaccine were determined. This was achieved by quantifying the polysaccharide specific antibody both *in vitro* and *in vivo*. They found that in the absence of *in vitro* stimulation PBMCs isolated after immunisation spontaneously secreted, polysaccharide specific antibody of all isotypes into the supernatants of the *in vitro* culture. This system revealed a peak in spontaneous antibody production on day 8

after immunisation. This was followed by a peak of *in vivo* serum anti-polysaccharide antibody by day 14(108). The data presented in this study describe a peak in plasma cell frequency by days 6-7 and this slightly precedes the peak of *in vitro* antibody seen in the Kehrl *et al* studies. This indicates that these authors were likely to be have been detecting the plasma cell response in the method used.

The reproducibility of the kinetics of circulating antigen specific plasma cells and serum antibody between different individuals in this and other studies suggests that these responses are a programmed physiological response to immunisation in a primed individual. Other published data indicate that these responses are not limited to Pnc7 as similar responses have been described after immunisation against tetanus and influenza (106, 374).

The spontaneously secreting plasma cells seen in the adult peripheral blood in this study are probably derived from memory B cells residing in secondary lymphoid tissues (145) as a result of the initial immunisation given in the booster group. The booster dose of Pnc7 then resulted in activation and rapid proliferation of vaccine specific, memory B-cells (260). By 6-7 days after immunisation there is a rapid efflux of antigen specific plasma cells into the peripheral blood, as was seen in this study, leading to enhanced protection from invasive disease (145, 256, 264).

The booster immunisation also induced significant levels of IgA-plasma cells in response to serotype 4, less so to serotype-23F and 14 and the diphtheria toxoid control. The peak of the IgA response matched that seen in the IgG response and both IgG and IgA may represent switched memory responses resulting from the first dose of Pnc7 administered to adults in the booster study, 12-17 months prior to boosting.

There was a low frequency of IgM-AFC detected post booster, that may represent plasma cells derived from the extrafollicular response of naïve B cells, B1 cells or MZB cells, that occurs in the first week after immunisation (126). Some of the IgM plasma cells may also arise from primary immune responses to capsular polysaccharides previously unseen by the donor. In the

primary immune response, B-cells activated by capsular polysaccharide differentiate into non-proliferating, IgM secreting short-lived plasma cells that secrete antibody with germline V-gene arrangements early in the immune response (145, 254-258) and disappear from the peripheral blood by two weeks after vaccination (259-262). Although the frequencies of IgM plasma cells described in this study were low, there was a trend towards a peak in the first week after the booster immunisation.

In previous studies it has been demonstrated that no memory B-cells are generated and no germinal centre reaction occurs in response to plain polysaccharide vaccines (256, 257, 263-266, 399, 413).

In human immunisation studies it has also been shown that repeat immunisation of adult humans with plain polysaccharide vaccines (4 months after a primary dose), does not induce a new peak of plasma cell activity or any rise in spontaneous antibody *in vitro* or serum antibody *in vivo*(108).

The data in this study, comparing the IgG plasma cell responses to a booster dose or a primary dose of Pnc7 suggests that re-immunisation with a pneumococcal conjugate vaccine does not significantly enhance the IgG plasma cell response to the capsular polysaccharides.

4.7.2 Immunisation with Pnc7 induced a rise in polysaccharide specific memory B cell frequency

Administration of a booster dose of Pnc7 in adults resulted in the increase frequency of IgG-AFC specific for serotypes 14 and 23F. Detection of these cells by ELISpot required *in vitro* stimulation of PBMCs by SAC+IL-2. The numbers of these polyclonaly induced IgG-AFC returned to pre-booster levels 1 month after the booster dose.

These observations suggest that memory B-cells induced by the primary immunisation 12-17 months earlier were mobilised to circulate in peripheral blood after the booster dose of Pnc7 was given. However, these cells may also be newly formed, non secreting plasmablasts that migrate

to the bone marrow before terminal differentiation into mature plasma cells. Many studies have shown that it is possible, by stimulation of peripheral blood cells by various combinations of polyclonal stimuli, to induce B cells to differentiate into IgG-ASC of various maturational stages. (183, 213, 372, 376, 377, 414).

ELISpot analysis of plasma cells *ex vivo* only allowed detection of fully differentiated plasma cells already secreting antigen specific antibody (415). Pre-existing, mature plasma cells only survive in culture for up to 48 hours and after 5 days stimulation with SAC+IL-2, only those B-cells entering culture as memory B-cells or plasmablasts remain (347). Analysis of B-cell division by flow cytometry and CFSE labelling has shown that five days of *in vitro* stimulation is sufficient for B cells to undergo at least 4 division cycles and that this number of division cycles is necessary for antibody secretion (260, 416, 417). This system preferentially activates memory B-cells (376, 377) and induces IgM memory B-cells ($CD27^{+}IgM^{+}IgD^{+}$) to secrete IgM and IgG, while class switched memory B-cells ($CD27^{+}IgM^{-}IgD^{-}$) will secrete IgG and IgA (267, 418, 419). The data from the present study indicated that high frequencies of IgM-AFC were formed following *in vitro* stimulation with SAC+IL-2, but minimal IgA-AFC were seen except in response to serotype 4. Also the frequency of IgM-AFC was constant through out the 15 days studied, which suggests that the IgM production was not specific to the vaccine antigen, particularly as there was equivalent amounts induced in response to the non-vaccine antigen control, tetanus toxoid. The SAC+IL-2 system probably induces low avidity, cross-reactive IgM that is secreted by B1 cell or MZB cell derived plasmablasts that fully differentiate in culture to secrete antibody.

Migration of the plasmablasts occurs over a limited period before they become resident in the bone marrow(254, 256, 257, 338), and this may explain the observed peaks in SAC+IL-2 induced cells at days 6-7 and the return to pre-booster levels of detectable IgG-AFC at 4-6 weeks post immunisation. The failure of this method to stimulate higher levels of memory B-cells to differentiate into IgG-AFC, at later time points maybe overcome in future work by the addition of

CpG-DNA and pokeweed mitogen to the culture medium (351, 370). Alternatively the numbers of pre-existing memory B-cells, detected in the peripheral blood may be maintained at a constant level (259).

In vitro stimulation of PBMCs with SAC+IL-2 also allowed the detection of memory B cell derived IgG-AFC that were of unrelated specificity to the vaccine (i.e. tetanus toxoid specific IgG-AFC). Although detectable throughout the study time course, the frequency of these cells remained steady, suggesting that IgG-AFC in the peripheral blood following immunisation were not only related to the administration of the Pnc7. There is evidence to suggest IgG-AFC of non-vaccine specificities appear in the peripheral blood as the result of displacement of long-lived plasma cells from the bone marrow or bystander activation of B-cells during an immune response (145, 254, 255, 257, 259, 263, 338, 347, 348, 351, 353, 355, 356, 358, 400, 401, 420, 421).

The migration of the plasmablasts and plasma cells is dependent on the expression of the chemokine receptor CXCR4 that has affinity for the chemokine CXCL12, secreted by bone marrow stromal cells (259). Up-regulation of plasma cells moving from the tonsils, through the blood to the bone marrow has been demonstrated in human(262). Once plasmablasts take up residence in the bone marrow, they terminally differentiate into mature plasma cells and lose the expression of CXCR4. In the event of displacement from their BM survival niche, the lack of CXCR4 expression renders these plasma cells incapable of returning from the periphery(257). It is thought that preference is given to newly arriving plasmablasts that still express the CXCR4 molecule, enhancing their ability to remain in the bone marrow environment (348). Another possibility is that CXCR4- plasma cells are re-circulating through the blood(262).

The data described here from the cell sorting studies based on CXCR4 expression, showed that on day 6 after immunisation CXCR4 was expressed on plasma cells of vaccine related specificities, but there were also plasma cells that did not express this chemokine receptor. This may be of relevance since the plasma cells at day 6 may have been a combination of both newly generated $CXCR4^{+}$ plasma cells and also pre-existing plasma cells, pushed out of bone marrow niches

because they no longer express CXCR4. An alternative theory is that they represented the long lived ($CXCR4^+$) migrating to the bone marrow and short lived $CXCR4^-$ plasma cells that re-circulate during the first week after immunisation (257, 259, 262, 348). CXCR5 is expressed by plasma cells homing back to the lymph nodes and it may be that some plasma cells re-circulate back to the lymph nodes to undergo further somatic hyper mutation in a second round of germinal centre development.

4.7.3 Serum antibody levels remained elevated following immunisation with Pnc7.

In the booster group, serum IgG specific for serotypes 14 and 23F remained elevated 12-17 months after the initial dose of vaccine and were enhanced following booster immunisation. However, there was no significant difference in the IgG concentration obtained in the booster group compared to the group who received only a primary dose. Therefore, although the number of doses did not appear to have an additive effect on antibody levels in adults, the antibody induced by Pnc7 was maintained for over 12 months.

The process behind maintenance of serum antibody is poorly understood. Three mechanisms have been suggested for the long term maintenance of protective antibody levels (145); presence of long-lived plasma cells (354, 358); bystander or polyclonal activation of B-cells (351); and re-stimulation of memory B-cells by antigen persisting on follicular dendritic cells (FDC) (422).

An alternative explanation in the case of pneumococcal antibody may be exposure to the organism through nasopharyngeal colonisation. Nasopharyngeal carriage of pneumococci was not investigated during this study but previous epidemiological studies, including one carried out in Oxford children, showed that 23F was among the most commonly detected serotype among nasopharyngeal isolates, while serotype-4 was the rarest and 14 was intermittently detected (58, 79, 92). Similar observations were made in a multinational epidemiological study of pneumococcal disease and carriage rates, (31). It is possible, therefore, that frequent exposure to

serotypes-14 and 23F, via nasopharyngeal carriage has helped to boost the AFC and antibody responses seen for 14 and 23F polysaccharides, generating a response that is predominantly IgG and due to pre-existing memory B-cell formation in exposed individuals. Conversely, little or no exposure to the infrequently colonising serotype 4 means that a more naïve response is generated following immunisation. This view is supported by data for the serotype 4 response, reported here, that showed no improvement in the magnitude of the response following the booster dose, although there was a switch to a more predominantly IgG response. In addition IgA and IgM were more apparent in the response to serotype 4 than in the immune response to serotypes 14 and 23F. These differences in antibody generation following immunisation are also reflected in higher rates of invasive pneumococcal disease caused by serotype 4 compared to 14 and 23F.

4.7.4 The phenotype of pneumococcal AFC

The plasma cell population in the peripheral blood on day 7 following immunisation included 2 subpopulations, $CD20^{+}CD27^{+}CD38^{+}Ig^{lo}$ and $CD20^{-}CD27^{+}CD38^{+}Ig^{lo}$. The $CD20^{-}$ cells are possibly terminally differentiated plasma cells expressing high (hi) levels of CD27 and CD38 with concomitant down regulation of CD20 and surface immunoglobulin (Ig) (183, 255, 259, 280, 358, 423). The $CD20^{+}$ subset probably represents intermediate plasmablasts with a more germinal centre phenotype, that are present in the peripheral blood and able to secrete antibody (263). The identification of the $CD20^{+}$ subset as a plasmablast population was further confirmed by the low level, surface expression of immunoglobulin. The upregulation of CD38 has been associated with differentiation into mature plasma cells (384) with tonsil and bone marrow plasma cell precursors expressing very low levels of CD38 (424). CXCR4 and CXCR5 populations may differentiate AFC populations migrating toward the bone marrow ($CXCR4^{+}$) (348, 386) and recirculating to the lymph nodes ($CXCR5^{+}$)(259). Plasmablasts migrate toward the CXCR4 ligand (CXCL12) expressed by bone marrow stromal cells(257, 348, 386) but mature plasma cells do not (386). Therefore the AFC detected at day 7 appear to be a mixture of newly

generated antigen specific (CXCR4+) plasma blasts leaving the lymphoid follicles for the bone marrow. Mature plasma cells are pushed out bone marrow niches into the blood, but are CXCR4-, therefore cant return to the bone marrow. This may explain the low frequency of tetanus toxoid specific cells seen in peripheral blood after immunisation with a heptavalent vaccine. CXCR5 expression by B cells is essential for follicular responses to occur (425). $CXCR5^{+}$ AFC may recirculate to the lymphoid tissues since CXCR5 knock-out mice show impaired class switching and antibody production in the long term(425).

$CD27^{+/-}IgM^{+}IgD^{+}$ B-cells were the predominant subset identified by flow cytometry in these adults. MZB express a similar phenotype of IgM^{hi} IgD^{lo} $CD21^{hi}$ $CD23^{lo}$, in the spleen and peripheral blood of humans and mice (253, 315). It has been suggested that MZB are involved in the early response to TI-2 antigens and produce AFC secreting large amounts of IgM antibody followed by rapid class-switching to IgG (267, 301, 323) and provide long lasting immunity (415, 426). MZB accumulate with age and have high frequencies of somatic gene mutations (144, 250, 252, 307, 320, 427). Some authors have suggested that, following the development of a functional splenic marginal zone in the second year of life, the improved efficacy of glycoconjugates over plain polysaccharide vaccines is reduced because polysaccharides are sequestered in the marginal zone even when conjugated to proteins, still inducing marginal zone like responses that are T-cell and CD40L independent (267, 315). These data from adults following immunisation with Pnc7 are consistent with this view.

The importance of the splenic marginal zone and germinal center for the formation of polysaccharide reactive plasma cells may explain the high risk of invasive pneumococcal disease in infants, who have immature germinal center structure (428) and lack MZB (253).

4.7.5 The magnitude of the immune response to Pnc7 is serotype dependent

There was considerable variability in the magnitude of the response against each antigen in a given individual and between subjects. This was true for the *ex vivo* plasma cell response, the *in vitro* stimulated, memory B cell response and the serum antibody response.

Individuals making good carrier specific responses (diphtheria,) did not necessarily make good polysaccharide specific responses and vice versa (188). The immunogenicity of serotype 23F, as measured by ex-vivo AFC frequency, was greater than that of serotype 4. This observation is supported by previous studies that measured IgG in serum (157, 165, 187). Previous studies investigating the effect of conjugation on polysaccharide specific responses have proposed that the individual polysaccharides conjugated to CRM_{197} provoke differing cytokine profiles within the germinal centres. This in turn affects the isotype and quality of antibody produced (189). Variations in immune responses to the same polysaccharide between individuals will likely depend on both host genetic factors and prior exposure to the organism through nasopharyngeal colonisation. However, differences in the polysaccharides themselves, particularly in relation to charge may direct significant differences in responses (429). The observation that T cell responses may after all be important in determining polysaccharide-specific B cell activation provides further possibilities to explain the variation in isotype response (131-134).

4.7.6 No difference in the B cell response was found following either a primary or booster dose of Pnc7

The benefits of administering a single or booster dose of Pnc7 was investigated in this study. Comparison was made between the groups based on plasma cell and plasmablast/memory B cell frequency and kinetics.

This revealed that there were no differences in plasmablast/memory B cell frequency following either a primary or booster dose of Pnc7. There were two exceptions to this observation however.

One was a trend for lower frequencies of anti-diphtheria IgG-AFC following the booster Pnc7 dose (p=0.06 at day 15 post immunisation). The second was for slightly higher IgG-AFC frequencies for serotype 23F at day 6 following a booster dose than after a primary dose (p=0.008). The general outcome of no difference is a result of great interest since there have been mixed views on the benefits of the use of conjugated pneumococcal vaccines in adults and more so in the elderly. This result follows that seen following re-immunisation with pneumococcal polysaccharide vaccines in the studies by Kehrl *et al* (108). In studies comparing the benefit of conjugate versus polysaccharide vaccine in adults, (101, 172) there appeared to be no real immunological benfit of re-immunisation with pneumococcal conjugate vaccines over polysaccharide vaccines. One study found a slight benefit of administering conjugate over polysaccharide, but that it was serotype dependent (410). While another found the benefits of conjugate vaccination were also age dependent and that conjugated and plain pneumococcal polysaccharide vaccines induced IgG2 preferentially over IgG1 in adults (172). Another study found that re-immunisation of adults with a pneumococcal conjugate, 6 months after the first dose, had no significant effect on the level of antibody generated (101). The poorer response to the CRM197 carrier protein (quantified here using diphtheria toxoid), may be explained following the study by Ralph Nanan *et al* (372), who showed that the frequency of IgG-AFC derived from *in vitro* culture of memory B cells was inversely proportional to the number of doses of diphtheria vaccine.

4.8 Conclusions

In conclusion it appears that in adults, the response to a booster dose of Pnc7 is marked by the generation of a population of rapidly responding plasma cells (IgG, IgA and to a lesser extent IgM,) that peaks at days 6-7 after immunisation. Memory cells generated following the response to the primary dose of Pnc7 rapidly differentiate upon re-encountering specific antigen, and spontaneously secret antibody in the ELISpot assay (106-108, 259, 264).

Culture with SAC+IL-2 allowed detection of IgG, IgA and IgM ASC, even after the *ex vivo* response had declined back to baseline.

The secreting cells were $CD20^{+/-}CD27^{+}CD38^{+}$ and expressed low levels of immunoglobulins. The $CD20^{+}$ plasmablasts resembled MZB with IgD and IgM expression and MZB are now considered to play a critical role in protective immunity against polysaccharide-encapsulated pathogens such as *S. pneumoniae*. The predominance of an IgM-ASC response in the cultured PBMC system may result from the activation of MZB-like $CD27^{+}CD20^{+}IgD^{+}IgM^{+}$ B-cells (124, 144, 252, 301, 415, 426, 430).

It is the aim of futher work to try and phenotype the cells that proliferate and differentiate into IgG-AFC following *in vitro* stimulation.

The speed of this immune response following encounter with pathogenic pneumococci may not be quick enough to prevent invasion of the organism in the first 6-7 days after acquisition in an individual with sub-protective levels of specific serum antibody. Generation of memory cells and long-lived plasma cells as detected in this study provides at least one mechanism to sustain serum antibody above protective thresholds and provide long-term protection against IPD.

Chapter 5: Identifying the B cell subsets involved in the humoral response to Pnc7

5.1 Abstract

The B cell response detected following *in vitro* stimulation of PBMCs with SAC+CpG+PWM (SCP) was compared with that seen following SAC+IL-2 (SI) stimulation. PBMCs were isolated on days 0, 6, 7, 15 and 28 after a primary dose of pnc7 in young adults.

This comparison revealed differences in the kinetics of the IgG-AFC response between the two culture systems. With SCP there was a peak in AFC frequency at day 15 post immunisation, while with SI the peak occurred on day 7 for vaccine related antigens. Non vaccine antigen (tetanus toxoid or total-IgG) specific IgG-AFC were detected at a constant frequency throughout the time course but were of much higher frequency in the SCP culture system.

Proliferation of B cell subsets was limited with SAC and PWM, but was enhanced by CpG in both the naïve and memory B cell populations. SCP induced the greatest proliferation and resulted in higher AFC frequencies than any individual stimulant. $CD27^{+}IgM^{+}$ B cells proliferated more strongly than $CD27^{-}IgM^{+}$ B cells. There was a rise in IgD^{+} B cell proliferation following immunisation, peaking at day 15 (IgD^{hi}) for SCP and day 28 (IgD^{hi}) for SI. These are classically naïve B cells while IgD^{lo} is more associated with memory B cells and this population peaked at day 6 following *in vitro* stimulation with SI.

The kinetics of proliferating B cells was not affected by the post immunisation time point chosen for analysis. The marker of a good proliferative response is >4 cell divisions as measured by CFSE dye dilution. Using SCP there was an accumulation of IgG^{+}, IgD^{lo} (memory) and IgD^{hi}

(naïve) B cells undergoing > 4 divisions after 5 days of culture. With SI only a small population of memory B cells proliferated past the >4 cell division cut off and no IgD^{hi} cells achieved this. Depletion of IgD^+ B cells appeared to remove the proliferation of naïve B cells (IgD^{hi}) but had no effect on the IgM^+CD27^+ B cell proliferation which included IgD^{lo} B cells. The IgD^+ fractions from the sorted B cells were enriched for proliferating naïve IgD^{hi} and IgD^{lo} IgM^+ memory B cells in comparison to that seen in the unsorted PBMC culture.

Conclusion

The difference in the IgG-AFC response seen following the *in vitro* stimulation of B cells with SAC+CpG+PWM and SAC+IL-2 rests with the B cell subsets differentiating during culture and is affected by the cells present in the peripheral blood at different time points after immunisation. SCP stimulated $CD27^+$, $CD27^-$, IgG^+, IgM^+IgD^+, IgM only, IgD^{lo} and IgD^{hi} B cells to differentiate into plasma cells. SI only stimulated IgG^+, IgD^{lo}, $CD27^+$ B cells to proliferate. AFC derived from naïve and memory B cells in the SCP system leads to increased IgG-AFC frequency in the ELISpot compared to SI.

The size of the proliferating population in the SI system is much smaller than seen for the same subsets in the SCP system. This is because SI only stimulates recently *in vivo* activated B cells and possibly plasmablasts to fully differentiate and they require fewer *in vitro* cell divisions to become mature plasma cells. SCP it truly polyclonal and will activate the entire subset of B cells and not just the antigen specific ones.

These studies have not clarified whether there is any effect of age on the generation of memory B cells and plasma cells or whether the type of antigen (conjugate or polysaccharide) might affect the response in each of the culture systems.

5.2 Introduction

To facilitate the detection of antigen specific AFC at time points that are distant from the time of antigen challenge requires the *in vitro* stimulation of B cells. The studies described so far have involved stimulation of PBMCs with a combination of *Staphylococcus aureus* Cowan Strain (SAC) and IL-2. This combination of B cell mitogen and cytokine has been used in many previously published studies (183, 213, 372, 376, 377, 414). These *in vitro* stimulatory conditions, plus or minus T cell factors such as IL-10 and anti-CD40-Mab, efficiently induce B cell proliferation and immunoglobulin secretion (mainly IgM and IgG), in cultures of PBMCs or purified B cells. However, the interest of the study described in this chapter was to see whether it would be possible to increase the sensitivity of the polysaccharide specific IgG-AFC ELISpot assay by using a combination of antigens reported by Crotty *et al* (370, 378, 431). They reported that a combination of SAC plus pokeweed mitogen (PWM) and CpG oligodeoxynucleotide (ODN) enabled the detection of smallpox specific memory B cells at more than 50 years after immunisation and that the frequency of the virus specific B cells increased following immunisation.

One substantial problem with trying to identify antigen specific memory B cells is that the majority of antigen specific memory B cells or long lived plasma cells are sequestered in physiological locations such as the bone marrow and secondary lymphoid organs which are not readily accessible. It has been shown that during immunological challenge with antigen that AFC and resting memory B-cells are induced to circulate through the peripheral blood and peak in frequency during the first two weeks following challenge. In some studies it has been suggested that the majority of circulating AFC are not related to the antigen of interest but are forced into the circulation by the arrival of newly generated memory and long lived plasma cells (254, 257, 400). As discussed in the preceding chapter it is possible to distinguish at least two subsets of circulating B cells following immunisation and to show the kinetics of arrival and disappearance

of these subsets in the circulation. There are at least two phenotypes of spontaneously secreting AFC. One of the subsets has a more mature plasma cell like appearance while the other has a more germinal centre like appearance (432).

Therefore it was of interest in this study to try and identify the B cell subsets that were involved in the response to glycoconjugate immunisation and whether it would be possible to detect different properties among these populations.

Resting naïve and memory B cells can be driven to proliferate and differentiate into plasma cells that secrete antibody by *in vitro* stimulation with polyclonal stimulants such as mitogens and also molecules that do not act via the BCR. By this process it is possible to detect small populations of re-circulating, antigen specific cells (by ELISpot), following their differentiation into AFC. The ability to detect such rare populations of B cells is particularly important in the absence of recent natural exposure to or immunisation with the antigen of interest. For example, the longevity of B cell memory to small pox immunisation, which may have occurred up to 50 years prior to the attempted detection of memory B cells, and for which there is no exposure to environmental antigen(370, 431, 433). In the case of the pneumococcus, there is a high rate of natural exposure to the organism via nasopharyngeal carriage, but any memory B cells that are specific for the capsular polysaccharides may be rare given the T cell independent nature of the antigen.

Traditional polyclonal B cell stimulants include pokeweed mitogen (PWM), *Staphylococcus aureus* Cowan Strain (SAC) and T-cells/T-cell supernatants/ factors such as interleukin (IL-)2. Individually these mitogens appear to stimulate distinct subsets of B cells.

5.2.1 Polyclonal stimulants of lymphocyte proliferation

PWM is a T-cell dependant (TD) mitogen that acts on T-cells, in co-culture with B cells, leading to provision of T cell help to the resting B cells. This T cell help may be through direct cell-cell

contact of in the guise of secreted cytokines. Removal of T cells from the system removes the activity of PWM on B cells (418, 434-436).

SAC has been described as a T cell Independent (TI) mitogen that is able to activate B cells by cross-linking surface bound immunoglobulin to generate an activation signal. In particular, the protein A component of the SAC cell wall (SpA) provides two separate mechanisms of B cell activation. One mechanism involves direct interaction of SpA with the surface immunoglobulin Fab fragments, cross-linking 2 or more B cell receptors (BCRs) to provide adequate signal strength for activation of B cells without T-cell help. The second mechanism involves SpA interation with the Fc portion of the surface bound Ig molecules (437-444).

T-cell factors, such as IL-2, act directly on activated B cells. IL-2 receptor (CD25) is up-regulated in activated B cell and increases the sensitivity of these cells to secreted IL-2 from activated T-cells(212, 213, 445). SAC in combination with IL-2 is a potent system for activation of B cells and their differentiation into antibody secreting plasma cells. SAC alone, while driving proliferation does not lead to differentiation of the B cells. Therefore, while proliferation is T-independent, differentiation is T-dependent. The combination of SAC+IL-2 has been used in a number of studies investigating human B cell activity (183, 213, 372, 376, 377, 414).

Other T cell factors involved in B cell activation and proliferation include direct interaction with antigen activated T cells via CD40-CD40L. In experiments using plasma membranes from activated helper T cell clones investigators have shown that murine B cells required CD40-CD40L interaction to induce B cell activation *in vitro* (419, 446-448). B cell receptor (BCR) ligation by T cell dependent antigens was enough to signal the B cell to up-regulate stimulatory molecules, such as CD40, CD80 and MHC class II (449, 450), but T cell help was needed for B cell differentiation and antibody class switching to occur (448). The resulting up-regulation of CD80 allowed the B cell to interact with CD28 on the T cell which in turn led to the up-regulation of CD40L on the T cell, which then interacted with CD40 on the B cell. This cognate interaction between B and T cell induced B cell proliferation. Proliferation is essential for B cell

differentiation into plasma cells. Blocking of this interaction in mouse experiments inhibited T cell dependent B cell activation (447). The strength of the interaction between CD40-CD40L and also concurrent exposure of activated B cells to various cytokines affected the isotype switching of antibody and survival of the B cells (448). Anti-CD40 Mab plus Th2 cytokines (such as IL-4 or IL-5) induced class switching from IgM to IgG1 (after 3 divisions), and IgE (after 5 divisions), shown in figure 5.1, while Th1 cytokines (such as IFNγ) induced switching to IgG2a. The isotype of antibody was also determined by the number of cell divisions undergone by the B cell (416, 451), and IFNγ was able to inhibit IL-4 mediated proliferation, decreasing the number of B cells undergoing cell divisions and thus altering the antibody isotype produced in culture (207, 452, 453).

CpG-ODN have recently been identified as good stimulators of lymphocyte stimulation. They are bacterial DNA fragments (or oligodeoxynucleotides,) containing unmethylated, cytosine-guanine (CpG) sequences that have been used as mitogens for human lymphocytes. CpG-ODN occur in two main types CpG-A and CpG-B that is determined by the nature of the link (p) between the C and G base (454). In CpG-A ODN the p-link is a phosphate bond and these sequences are efficient activators of natural killer (NK) cells and IFNγ producing dendritic cells. In the CpG-B ODN sequence, the p-link is a phosphorothioate bond and these sequences are potent stimulators of human B cells (455). CpG-B ODN motifs are surrounded by specific base sequences and it was found that optimal activity required the presence of **TpC** base pair 5' of three CpG (5'-GTCGTT-3') motifs that were separated by TpT. Alone or in combination with SAC and or PWM and IL-2, CpG is able to activate B cells via the toll like receptor (TLR-)9 (456, 457). TLR-9 is up-regulated on activated and memory B cells and provides a mechanism for provision of a second signal for B cells activated via the antigen receptor. The most potent ODN sequence for activating human B cells was identified as (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') and given the name ODN 2006 (231, 232, 458).

Combinations of these mitogens act synergistically to drive the maximal B cell proliferation *in vitro*, allowing expansion and identification of antigen specific memory B cells many years after immunisation (228, 351, 359, 370, 371, 459).

In the initial pilot vaccine studies in chapters 3 and 4 B cells were stimulated with a combination of SAC+IL-2 and while finding memory B cell responses in adults the sensitivity of detection in infants appeared far lower. Therefore, to enhance the sensitivity of detection in the Elispot assay the effects of two culture systems on the peripheral blood of adults were compared. The first was the original culture system of SAC+IL-2 (SI) and the second was one published by Crotty *et al* (378), using a combination of SAC+CpG+PWM (SCP).

The comparison involved labelling adult PBMCs with CFSE and then culturing the cells with either SCP or SI for 5 days. CFSE labelling allowed the number of cell divisions undergone by the PBMCs (and individual B cell subsets), in each of the culture conditions to be determined as cell division sequentially dilutes the fluorescent dye (fig.5.1). As discussed above it has previously been shown that B cells need to undergo at least 4 cell divisions before immunoglobulin secretion occurs (387, 416, 417, 451) and it is thought that T-cell help, either through cognate interaction or via T-cell factors, is required for the differentiation of B cells into antibody forming cells (AFC)(207, 450, 451, 460).

Therefore, the aim of this study was to determine whether the *in vitro* stimulation of PBMCs with SAC+CpG+PWM compared to SAC+IL-2 would increase the frequency of IgG-AFC detected by ELISpot. It was also the aim of this study to determine whether distinctions could be made between the proliferating B cell subsets within each of the *in vitro* stimulation systems and whether this related to the ELISpot outcome.

5.3 Subjects and clinical procedures

A group of 10 adults with no previous pneumococcal vaccination history were recruited with informed consent and after ethical approval (OxRec C02.005), to receive a single dose of Pnc7.

Blood was drawn on days 0, 6, 7, 15 and 28 following immunisation with a single dose of Pnc7 vaccine (Chapter 2, section 2.4). Serum was stored at -80°C and PBMCs were isolated from 18ml of heparinised whole blood.

Two unimmunised, adult volunteers were recruited from the laboratory with informed consent to isolated PBMCs to evaluate the cell culture conditions for the memory B cell ELISpot assay described in figures 5.6 – 5.8.

5.4 Methods

PBMCs were isolated as described in Chapter 2, section 2.4 at each of the time points (day 0, 6, 7, 15 and 28).

In order to increase sensitivity of the memory B cell ELISpot assay the original stimulatory antigen combination of SAC+IL-2 (SI) was compared with a new combination, SAC+CpG+PWM (SCP), suggested by Crotty *et al* (378).

Equal numbers of PBMCs from each sample (a total of $9.6x10^6$ cells at $2x10^5$cells/well), were stimulated with either SI or SCP for five days at 37°C in 5%CO_2 and 95% humidity (as in Chapter 2, section 2.6).

Prior to *in vitro* stimulation with SI or SCP the PBMCs were labelled with 2μM CFSE (as in Chapter 2, section 2.14), to assess cellular proliferation by dye dilution on a flow cytometer. By using CFSE dye dilution analysis it was possible to look for differences in proliferative capacity of different B-cell subsets following *in vitro* stimulation (figure 5.1).

After 5 days the cells were harvested and labelled with combinations of CD19-PerCP, IgD-PE, IgG-PE, IgM-Cychrome and CD27-PE, for analysis of proliferating B cell subsets by flow cytometry (as in figure 5.1). The remaining harvested cells were seeded in antigen coated plates for enumeration of diphtheria, tetanus, serotype 4, 14 and 23F specific IgG-AFC by ELISpot

(Chapter 2, sections 2.5, 2.7, and 2.8). The phenotypes of the proliferation B cell populations and all the Ig-secreting activity were compared between the two culture conditions.

Fresh PBMCs isolated on day 7 or 28 following immunisation were separated using AutoMACs^{TM} magnetic cell separation, on the basis of the surface expression of IgD or IgG as in (Chapter 2, section 2.9).

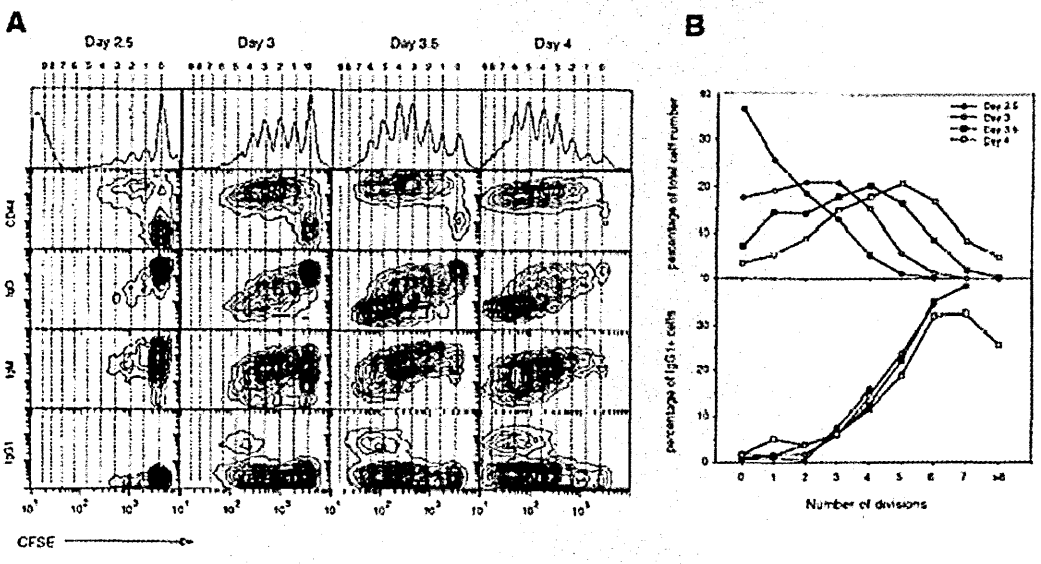


Figure 2. Tracking B cell division, isotype switching, and differentiation using CFSE. CFSE-labeled small, dense B cells were stimulated with Th membrane and in for various times before being harvested and stained. (*A*) (*Top*) CFSE fluorescence histograms for activated B cells taken at days 2.5, 3, 3.5, and 4 after the start of culture. The progression of cell division with time is indicated by sequential twofold reductions in CFSE intensity. Dashed guidelines on each graph indicate the position of each division cycle number. Underneath each histogram are two-dimensional contour plots of CFSE versus surface labeling with CD44, IgD, IgM, and IgG1. (*B*) Data from *A* were gated to calculate the proportion of total viable cells in culture found in each division cycle at each time point (*top*). (*Bottom*) The proportion of cells in each division cycle that were IgG1$^+$ at each time point. Adjacent gates were set using Cellquest software to accommodate sequential division rounds centered on the mean for each division.

Reference: B-cell differentiation and isotype switching is related to division cycle number. Hodgkin PD, Lee JH, Lyons AB. J. Exp Med. 1996 Jul 1;184(1):277-81

Figure 5.1 Division cycle number and B cell surface phenotype following *in vitro* stimulation with Th plasma membranes.
The example in this figure is from a study by Hodgkin *et al*, and shows how the number of dividing cells can be established for each of the B cell subsets and how proliferation affects the expression of surface immunoglobulin.

5.5 Results

5.5.1 Kinetics and frequency of IgG-AFC derived from adult memory B-cells following 5 days of stimulation with SCP (blue) versus SI (red).

Using SCP to stimulate PBMCs induced a significantly higher frequency of total IgG-AFC than that generated following PBMC stimulation with SI and this remained so throughout the time course (Fig. 5.2a, p=<0.01 at days 0, 6 and 7; p=<0.05 at days 15 and 28). The frequency of tetanus toxoid specific IgG-AFC was a significantly higher following PBMC stimulation with SCP than with SI at all of the time points except at day 0 (Fig 5.2b. p=<0.01.) The frequencies of both the total-IgG-AFC and the tetanus toxoid specific IgG-AFC remained constant throughout the time course irrespective of the type of stimulus used *in vitro* (fig. 5.2a-b.)

Stimulation of PBMCs with either SI or SCP generated similar frequencies of diphtheria specific IgG-AFC at all time points throughout the time course (Fig.5.2c). There was a difference in the kinetics between the two *in vitro* systems however. In the SI system the frequency of inducible diphtheria toxoid specific IgG-AFC peaked above baseline levels on day 7 following immunisation (p=<0.01, red asterix.) Numbers remained elevated above baseline at day 15 but had returned to baseline by day 28 (fig 5.2c.) The median frequency of diphtheria specific IgG-AFC induced by SCP stimulation peaked at day 15 following immunisation (p=<0.01, blue asterix), but had returned to baseline by day 28 (fig.5.2c).

Prior to immunisation the *in vitro* stimulation of PBMCs with SCP induced significantly higher frequencies of IgG-AFC specific for the polysaccharide capsules of serotype 4 and 23F than was seen following stimulation with SI (p=<0.01, black asterix, fig.5.2d+f). No difference was seen in the baseline frequency of serotype 14 polysaccharide specific IgG-AFC between the two systems (fig.5.2e).

Following immunisation there was a rise in frequency of polysaccharide specific IgG-AFC induced by both the SCP and SI *in vitro* stimulation systems. The elevation in frequency was first apparent at day 7 and tailed away again by day 28 (fig.5.2d-f).

The post immunisation frequencies of polysaccharide specific IgG-AFC induced by *in vitro* stimulation of PBMCs with SCP were equal to or greater than those seen following stimulation with SI at day 7 and were significantly higher than those induced by SI at days 15 and 28 in response to serotypes 4, 14F ($p=<0.01$) and 23F ($p=<0.05$) (fig.5.2d-f, black asterix).

The median frequency of IgG-AFC induced by *in vitro* stimulation of PBMCs with SCP peaked significantly at day 15 for serotypes 4, 23F ($p=<0.01$), and 14 ($p=<0.05$) (fig.5.2d-f, blue asterix). By day 28 post immunisation only serotype 4 specific IgG-AFC remained significantly elevated above baseline in the SCP system ($p=<0.05$, fig.5.2d, blue asterix).

The median frequency of IgG-AFC induced by *in vitro* stimulation of PBMCs by SI peaked significantly above baseline at day 7 for serotype 4 ($p=<0.01$), and 23F ($p=<0.05$) and remained elevated above baseline for serotype 23F at day 15 ($p=<0.05$) (fig.5.2d-f, red asterix).

Thus these data show that SCP induces higher frequencies of IgG-AFC from B cells than does SI if B cells have not recently been stimulated by the immunising antigen (e.g. tetanus toxoid specific IgG-AFC). IgG-AFC specific for recent vaccine antigens (diphtheria toxoid and the pneumococcal polysaccharides), were detected at similar frequencies in both the SCP and SI systems from PBMCs isolated in the first week after immunisation. Higher frequencies of polysaccharide specific IgG-AFC were seen at baseline and day 15 to 28 following *in vitro* culture with SCP then with SI. This suggested that there were differences in the B cells present at these time points.

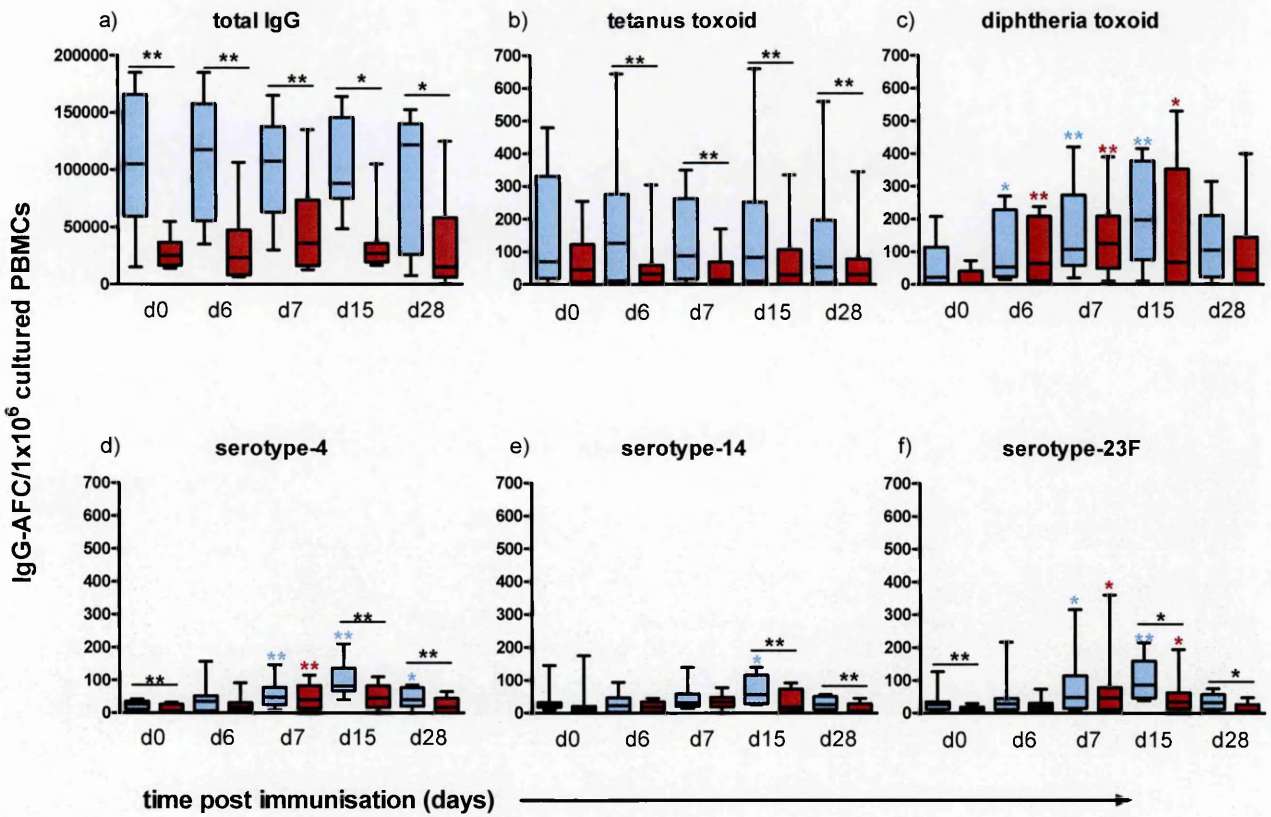


Figure 5.2 Comparison of memory B cell derived IgG-AFC frequency following *in vitro* stimulation of PBMCs with SAC+IL-2 (SI) or SAC+CpG+PWM (SCP).

Adult PBMCs were isolated on days 0, 6, 7, 15 and 28 and stimulated, *in vitro*, with either SCP (blue) or SI (red) for 5 days. The cultured cells were then harvested and allowed to secrete antibody overnight onto ELISpot wells coated with anti-human IgG, diphtheria and tetanus toxoids and pneumococcal polysaccharides 4, 14 and 23F. (**p=<0.01, *p=<0.05, colour (blue/red) coded to show increase above baseline within each culture system and black to show differences between culture systems.

5.5.2 CFSE dye dilution analysis of B-cell subset proliferation

PBMCs were isolated from an un-immunised adult donor to determine the effect of the different polyclonal stimulants either individually or in combination and which induced the best proliferation of lymphocytes. The stimulants used were 10%-NBBS only, SAC (1/5000), PWM (83ng/ml), CpG (2.5µg/ml), SAC+CpG, SAC+PWM, PWM+CpG and SAC+CpG+PWM.

Following 5 days of culture the cells were harvested and labelled for surface expression of IgM-PerCP and CD27-PE with CFSE providing the third colour for analysis by three colour flow cytometry. The cells were first gated on the live lymphocyte population (fig 5.3a).

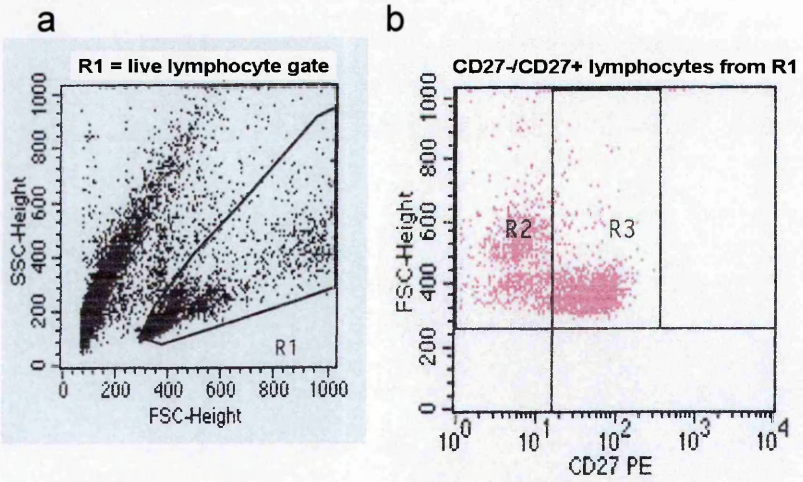


Figure 5.3 An example of the lymphocyte gating used prior to CFSE proliferation analysis of PBMC samples that had undergone 5 days of *in vitro* stimulation.
(a) Live lymphocyte gate, R1 and (b) shows the subgates for $CD27^-$ (R2) and $CD27^+$ (R3) lymphocytes within R1 population.

Within the R1 population the overall lymphocyte proliferation was analysed (fig.5.4a-h) for each of the *in vitro* stimulants alone and in combination. The PBMCs were labelled with CFSE and cultured for 5 days in the presence of CpG, SAC, PWM or combinations of these as shown in figure 5.4.

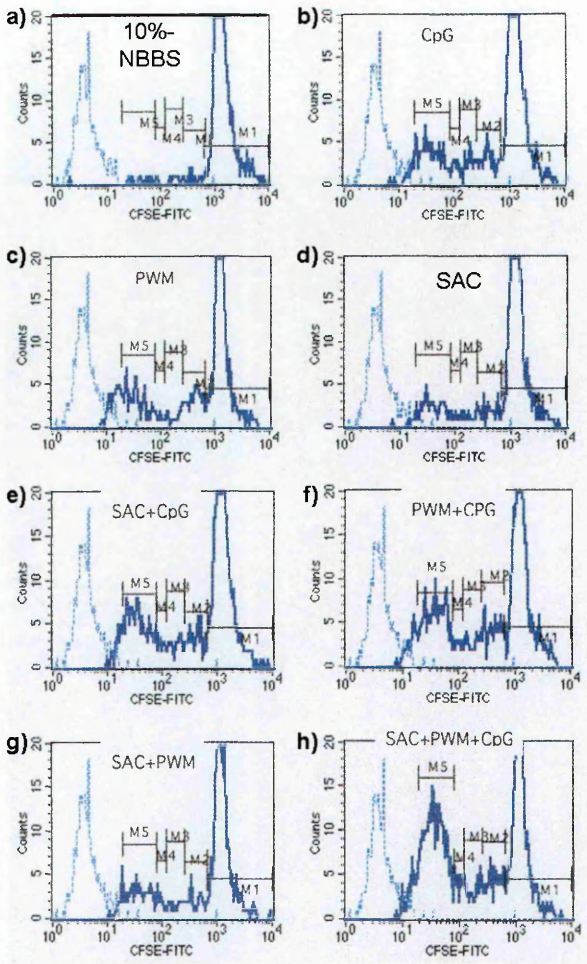


Figure 5.4 Total CFSE proliferation of PBMCs in R1 (fig 5.3a) following 5 days of *in vitro* stimulation with individual and combinations of polyclonal B cell activators (day 28 post immunisation).
Isolated PBMCs were labelled with 2μM CFSE and then cultured for 5 days with a) 10%NBBS alone (unstimulated control, M1), b) CpG 2.5μg/ml, c) PWM (83ng/ml), d) SAC (1:5000) or combinations of these; e)SAC+CpG, f) PWM+CpG, g) SAC+PWM and f) SAC+CpG+PWM. After 5 days of stimulation the cells were harvested and the total lymphocyte population was analysed for the amount of proliferation by CFSE dye dilution (dark blue line). The negative cut off for CFSE fluorescence was made using the unlabelled cell controls (pale blue line). The markers (M1-M5) represent 0, 1, 2, 3 or ≥4 cell divisions based on reduction in CFSE fluorescence (x-axis) and cell numbers (y-axis).

Of the three individual stimulants it can be seen that CpG (5.4b) and PWM (5.4c) induced more lymphocytes to divide ≥4 times than did SAC alone (5.4d). When the stimulants were combined there appeared to be a synergistic effect, with all combinations inducing more cell division than any of the individual stimulants alone. Combining SAC with either CpG (fig.5.4e), or PWM

(fig.5.4g) increased the number of cells undergoing ≥4 cell divisions after 5 days of culture when compared to SAC alone (fig.5.4e and g).

The combinations that included CpG (SAC+CpG, PWM+CpG, fig 5.4e-f) appeared to induce more cell division than was seen in response to stimulation with SAC+PWM (5.4g.) The best combination was SAC+CpG+PWM (fig.5.4h), as had been suggested by Crotty *et al* (378).

The percentage of CFSE positive lymphocytes within each marker (M1-M5) was then plotted for each of the culture conditions and is shown in figure 5.5. The 10%-NBBS control induced minimal background proliferation of lymphocytes with 99% of lymphocytes remaining undivided. In response to SAC stimulation 85% of lymphocytes remained undivided, followed by CpG (78%), and PWM (68%). With the combined stimulants the percentage of undivided lymphocytes was greatest in response to SAC+PWM (72%), followed by SAC+CpG (65%), then PWM+CpG (58%) and finally SAC+CpG+PWM where only 43% of lymphocytes remained non-proliferating.

The percentage of CFSE positive lymphocytes achieving 4 or more divisions was greater in response to SAC+CpG+PWM (35%) followed by PWM+CpG (21%), SAC+CpG (18%), SAC+PWM (14%), PWM (12%), CpG (10%) and SAC (8%).

For a more detailed analysis of the B cell subsets proliferating during the 5 day stimulation culture a further sub analysis was carried out on the memory B cell ($CD27^+$), and naïve B cell ($CD27^-$) populations, focusing on the surface expression IgM. For this analysis the lymphocytes (fig.5.3a), were sub-gated on the absence (R2) and presence (R3) of surface CD27 expression (Fig 5.3b). Within the $CD27^+$ and $CD27^-$ lymphocyte populations the B cells were selected for the surface expression of IgM and dot plots were created showing the $CD27^{+/-}$ IgM^+ populations versus CFSE dilution (fig.5.6a-p).

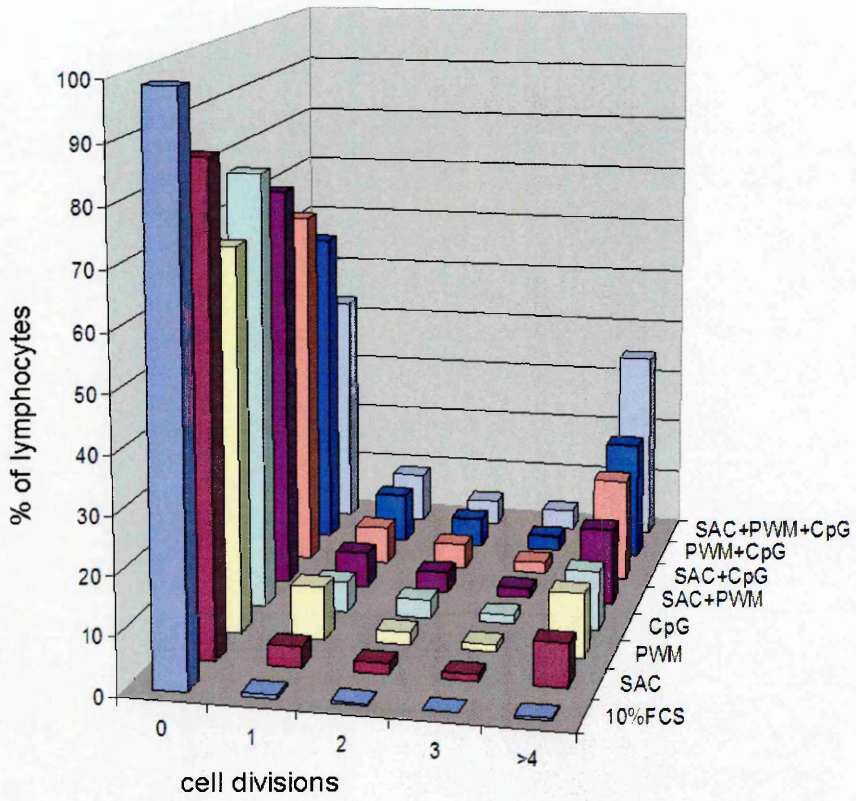


Figure 5.5 Comparison of proliferative capicity of B cells following 5 days *in vitro* stimualtion with different combinations of polyclonal activators.

PBMCs isolated 28 days after a primary dose of Pnc7 were labelled with 2μM CFSE and then cultured for 5 days with 10%NBBS alone (purple, unstimulated control,) CpG 2.5μg/ml (green), PWM 83ng/ml (yellow), SAC 1:5000 (red), SAC+CpG (orange), PWM+CpG (blue), SAC+PWM (dark purple) and SAC+CpG+PWM (lilac). After 5 days of stimulation the cells were harvested and the total lymphocyte population (figure 5.2a) was analysed for the amount of proliferation by CFSE dye dilution. The data in this figure is a graphical representation of the data in figure 5.4. The percentage of lymphocytes (y-axis) within each of the marker bounds (M1-M5) shown in figure 5.4 plotted against the number of cell divisions (0, 1, 2, 3, ≥4) for each of the culture conditions.

In the un-stimulated controls (10%-NBBS only, fig.5.6a+e), there remained a surviving population of non-dividing, naïve ($CD27^-$) IgM^+ cells (fig.5.6a), while no non-dividing memory ($CD27^+$) IgM^+ lymphocyte population remained (fig.5.6e).

Stimulation of B cells with SAC or PWM appeared only to induce proliferation of $CD27^+IgM^+$ memory B cells (fig.5.6.f-g), but had no effect on the naïve $CD27^+IgM^+$ B cell population (fig.5.6b-c).

CpG stimulation, however, induced proliferation of both naïve (fig.5.6d), and memory (fig.5.6h), B cells although the number of cell divisions undergone by the naïve B cell population was lower than in the memory B cell population.

An x-axis (log^{10} CFSE fluorescence), value of $8x10^1$ marked the ≥ 4 cell division marker as denoted in figure 5.4. While CpG induced the majority of $CD27^+IgM^+$ memory B cells to proliferate past this point, the majority of the naïve $CD27^-IgM^+$ B cell population also proliferated but did divide as quickly as the memory B cells within the same time frame. Therefore CpG was able to induce proliferation of both naïve and memory B cells.

Combining SAC+PWM had an additive effect on the rate of proliferation of memory B cells (fig.5.6m), when compared to either mitogen alone but did not alter the naïve B cell response (fig.5.6i). The combination of CpG with either SAC (fig.5.6j+n), or PWM (fig.5.6k+o), enhanced the proliferative response of both the naïve and memory B cell subsets when compared to any of the three stimuli alone. Combining SAC+CpG+PWM induced the majority of both naïve (fig.5.6l), and memory (fig.5.6p), B cells to undergo ≥ 4 cell divisions.

Thus SAC+CpG+PWM appeared to be the ideal combination of mitogens for the stimulation of B cells for the memory B cell ELISpot.

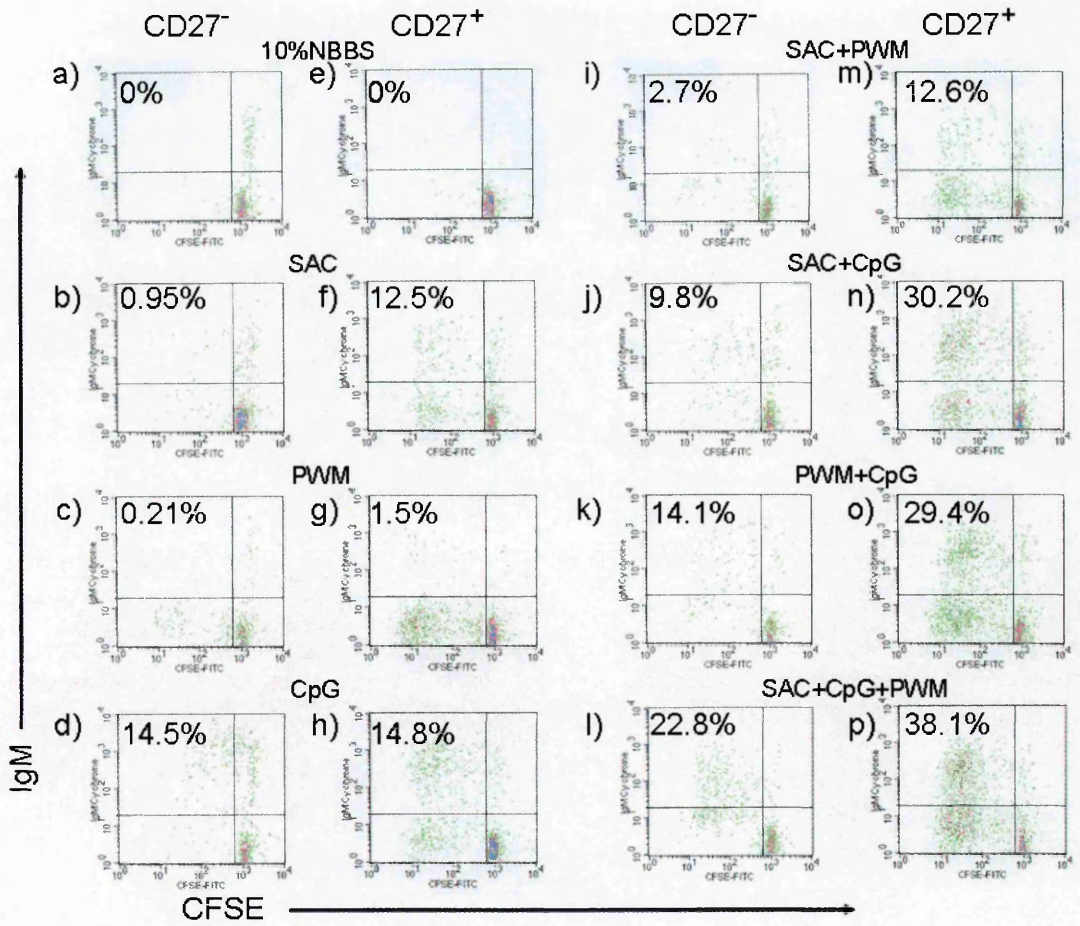


Figure 5.6 CFSE proliferation of $CD27^-$ (a-d, i-l) or $CD27^+$ (e-h, m-p) B cell subsets positive for IgM^+ expression.
PBMCs from a healthy adult donor were stimulated for 5 days with (a+e) 10%NBBS, (b+f) SAC, (c+g) PWM, (d+h) CpG, (i+m) SAC+PWM, (j+n) SAC+CpG, (k+o) PWM+CpG and (l+p) SAC+CpG+PWM. The data represents the number of $CD27^-$ or $CD27^+$ B cells expressing IgM^+ (y-axis) against the CFSE fluorescence (x-axis) with proliferating cells moving towards the left along the x-axis.
The upper left quadrant shows the percentage of B cells that are proliferating and the upper right quadrant show the non-dividing B cells.

5.5.3 Immunoglobulin secretion by *in vitro* stimulated, peripheral blood B cells

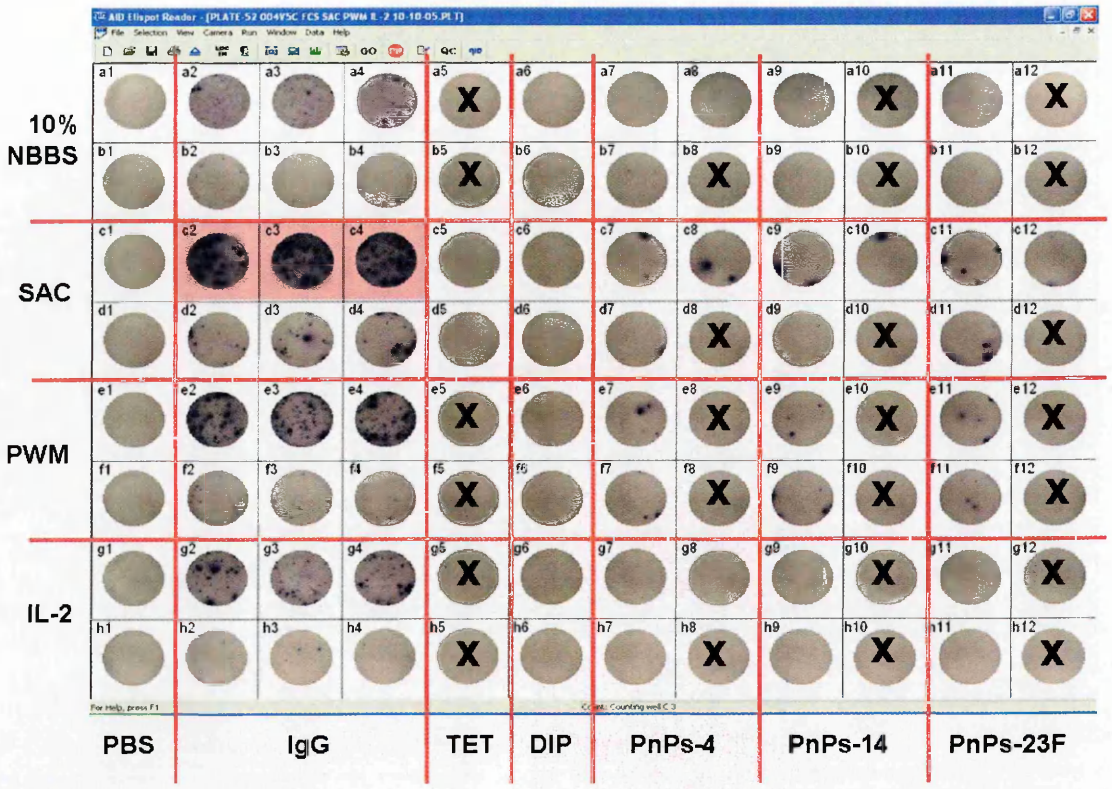
PBMCs isolated on day 28 after a single dose of Pnc7 vaccine and cultured for 5 days with SAC, PWM, CpG, IL-2 (50U/ml) and SAC+CpG+PWM were harvested and seeded into antigen specific ELISpot plates for the detection of IgG-AFC or IgM-AFC as shown in figures 5.7 and 5.8.

There was some residual total IgG-AFC activity from the un-stimulated cells (fig.5.7 10%-NBBS) but no antigen specific activity.

Stimulation with SAC or PWM alone (fig.5.7), induced more of both total IgG-AFC and also antigen specific IgG-AFC with very similar numbers of pneumococcal capsular polysaccharide specific AFC for serotypes 4, 14 and 23F. There were no tetanus or diphtheria specific AFC however. Stimulation with IL-2 induced some total-IgG-AFC secretion but no antigen specific AFC were seen. CpG alone also appeared as equally effective at inducing the formation of IgG-AFC as SAC and PWM alone (fig.5.8, rows g and h). CpG also appeared to induce tetanus toxoid specific IgG-AFC formation, while no tetanus or diphtheria specific AFC were seen in response to SAC or PWM.

The combination of SAC+CpG+PWM strongly induced IgG-AFC formation to all three capsular polysaccharides, tetanus and diphtheria toxoid (fig.5.8, rows a-c), and also an overwhelming IgM-AFC response (fig.5.8, rows d-f). In fact, when paired with the CFSE proliferation data from figure 5.6 it seems that the SAC+CpG+PWM stimulation system preferentially induces IgM production in comparison to IgG.

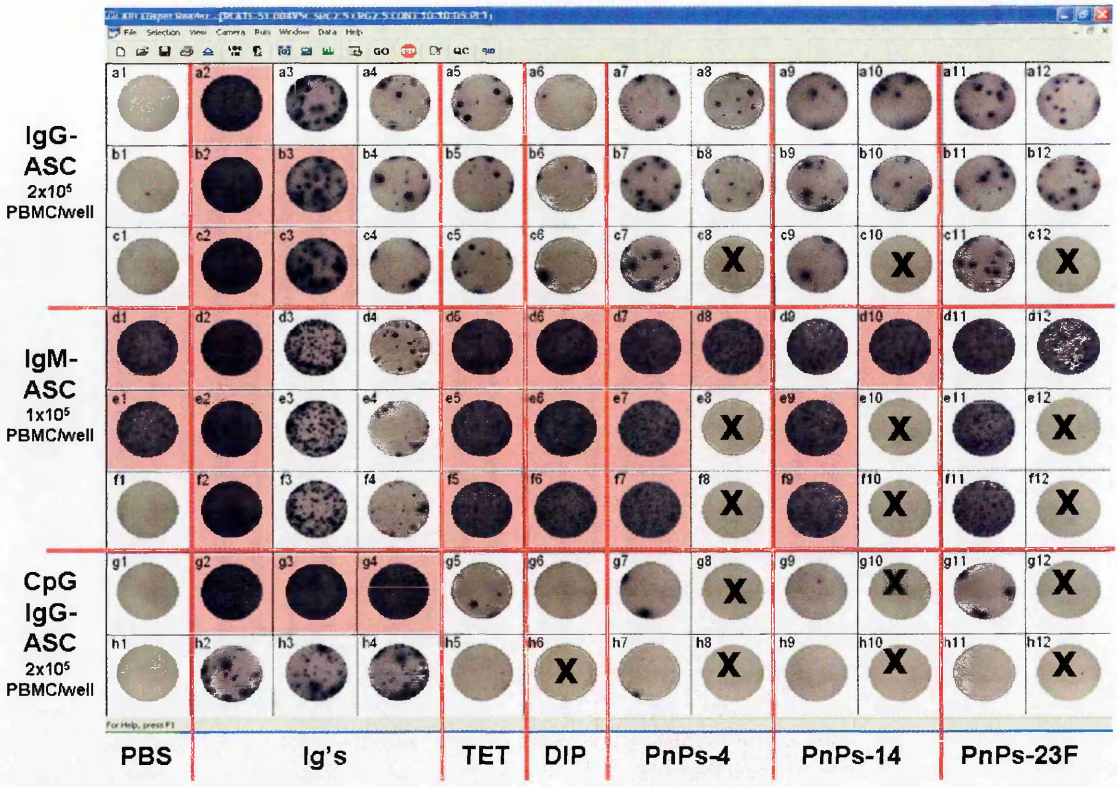
These experiments were only carried out on individual donors, but provided valuable information about the effects of the individual and combined polyclonal stimulants.



Template		1	2	3	4	5	6	7	8	9	10	11	12
10%NBBS	A	PBS	1:10	1:10	1:10		dip	4	4	14		23F	
$2x10^5$cells/well	B	PBS	1:100	1:100	1:100		dip	4		14		23F	
SAC	C	PBS	1:10	1:10	1:10	tet	dip	4	4	14	14	23F	23F
$2x10^5$cells/well	D	PBS	1:100	1:100	1:100	tet	dip	4		14		23F	
PWM	E	PBS	1:10	1:10	1:10		dip	4		14		23F	
$2x10^5$cells/well	F	PBS	1:100	1:100	1:100		dip	4		14		23F	
IL-2	G	PBS	1:10	1:10	1:10		dip	4	4	14		23F	
$2x10^5$cells/well	H	PBS	1:100	1:100	1:100		dip	4		14		23F	

Figure 5.7 The ELISpot (IgG) output of from PBMCs stimulated with 10%NBBS, SAC, PWM or IL-2 (50U/ml).

PBMCs were isolated from a healthy adult donor and stimulated for 5 days. The cells were then harvested, re-suspended to the cell concentrations shown in the left hand columns of the template shown above and seeded into well of an ELISpot plate coated with PBS, anti-Ig (columns 2-4), tet, dip, serotype 4, 14 and 23F. The cells for the Ig-control wells were diluted 1:10 and 1:100 from the initial cell suspension. The secreting cells were detected with anti-IgG-alkaline phosphatase conjugate. The black crosses show wells that received no cells due to sample volume constraint.



Template		1	2	3	4	5	6	7	8	9	10	11	12
SAC+CpG+PWM IgG-AFC 2x10^{5}cells/well	A	PBS	1:10	1:100	1:1000	TET	DIP	4	4	14	14	23F	23F
	B	PBS	1:10	1:100	1:1000	TET	DIP	4	4	14	14	23F	23F
	C	PBS	1:10	1:100	1:1000	TET	DIP	4		14		23F	
SAC+CpG+PWM IgM-AFC 1x10^{5}cells/well	D	PBS	1:10	1:100	1:1000	TET	DIP	4	4	14	14	23F	23F
	E	PBS	1:10	1:100	1:1000	TET	DIP	4		14		23F	
	F	PBS	1:10	1:100	1:1000	TET	DIP	4		14		23F	
CpG2.5µg/ml 2x10^{5}cells/well	G	PBS	1:10	1:10	1:10	TET	DIP	4		14		23F	
	H	PBS	1:100	1:100	1:100	TET		4		14		23F	

Figure 5.8 The ELISpot output and template for PBMCs isolated from the same donor as in figure 5.7.

The PBMCs were stimulated *in vitro* with SAC+CpG+PWM, or CpG alone for 5 days. The cells were then harvested, re-suspended to the cell concentrations shown in the left hand column of the template shown above and seeded into well of an ELISpot plate coated with PBS, anti-Ig (columns 2-4), tet, dip, serotype 4, 14 and 23F. The cells for the Ig-control wells were diluted 1:10 and 1:100 from the initial cell suspension (and 1:1000 for SAC+CpG+PWM). The secreting cells were detected with either anti-IgG-alkaline phosphatase conjugate (rows a-c and g-h) or anti-IgM-alkaline phosphatase conjugate (rows d-f). The black crosses show wells that received no cells due to sample volume constraint.

5.5.4 The total IgD^+, IgM^+ and IgG^+ B cell subset *in vitro* proliferation after Pnc7 immunisation. (SAC+IL-2 vs SAC+CpG+PWM)

The aim of this study was to determine the ideal conditions with which to generate IgG-AFC from memory B cells. CFSE dye dilution analysis enabled the identification of the B cell subsets involved in the response to Pnc7 immunisation.

Adult donors received a single dose of Pnc7 and PBMCs were obtained on days 0, 6, 7, 15 and 28 after immunisation. The PBMCs were labelled with CFSE and cultured with SAC+IL-2 (SI) or SAC+CpG+PWM (SCP) for 5 days. Following *in vitro* stimulation the cells were harvested and labelled for expression of surface immunoglobulin. The ability of B cells from the immunised donors to proliferate in response to polyclonal stimulation at various time points after vaccination was quantified (fig 5.9).

The proliferation of IgD^+ B cells (fig.5.9a), peaked above baseline by 7 to 15 days after immunisation This was significant in the SCP system ($p=0.031$), but in the SI system the increased proliferative response was delayed until days 15 to 28 ($p=0.011$, $p=0.018$ respectively). IgD^+ B cell proliferation at day 6 post immunisation was significantly lower than at day 0 in the SCP system ($p=0.009$), while no such effect was seen in the SI system. There was no appreciable alteration in the ability of IgM^+ B cells (fig.5.9b), to proliferate in response to either set of stimuli at any of the time points studied. There was no change in the *in vitro* proliferation of IgG+ B cells isolated after immunisation (fig 5.9c).

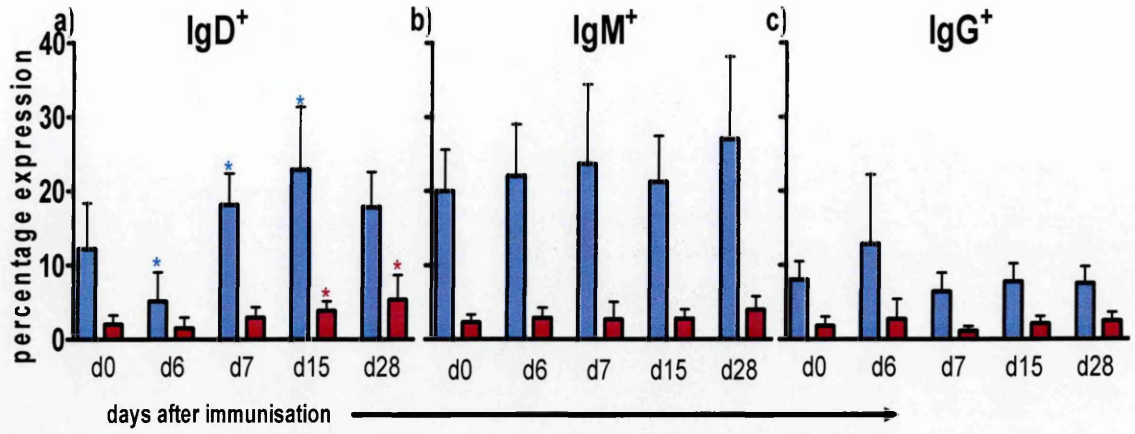


Figure 5.9 The total amount of proliferation (>1 cell division in 5 days), within the IgD^+, IgM^+ or IgG^+ B cell subsets.
PBMCs were isolated from adult volunteers on days 0, 6, 7, 15 and 28 after a single dose of Pnc7 and labelled with 2μM CFSE. The labelled PBMCs were the cultured for 5 days in the presence of SAC+CpG+PWM (blue) or SAC+IL-2 (red). On day 5 of stimulation the cells were harvested and labelled for expression of surface immunoglobulins. The data represent the percentage of a) IgD^+, b) IgM^+ and c) IgG^+ B cells undergoing at least one CFSE dye dilution, (or cell division), at the end of 5 days. The columns represent the mean of 10 individuals, with 95% CI, */*p=<0.05.

5.5.5 The effect Pnc7 immunisation on the frequency of IgD^+ B-cells following *in vitro* stimulation

Since Pnc7 immunisation appeared only to affect the proliferation of IgD^+ B cells the overall frequency of IgD expressing B cells was assessed following *in vitro* stimulation of the isolated PBMCs (fig.5.10a-d).

In the SCP system IgD^{hi} expressing B cells reached a peak in frequency by day 15 (p=0.045, fig.5.9a), after immunisation while the percentage of the IgD^{lo} subset remained constant throughout the time course (fig.5.10b).

Following stimulation of PBMCs with SI the IgD^{hi} B cells remained at a constant level until day 28, by which time the frequency of proliferating IgD^{hi} B cells was elevated significantly above baseline (p=0.042, fig.5.10c). The IgD^{lo} B cells peaked above baseline on day 6 (p=0.017, fig.5.10d), and then returned to baseline levels.

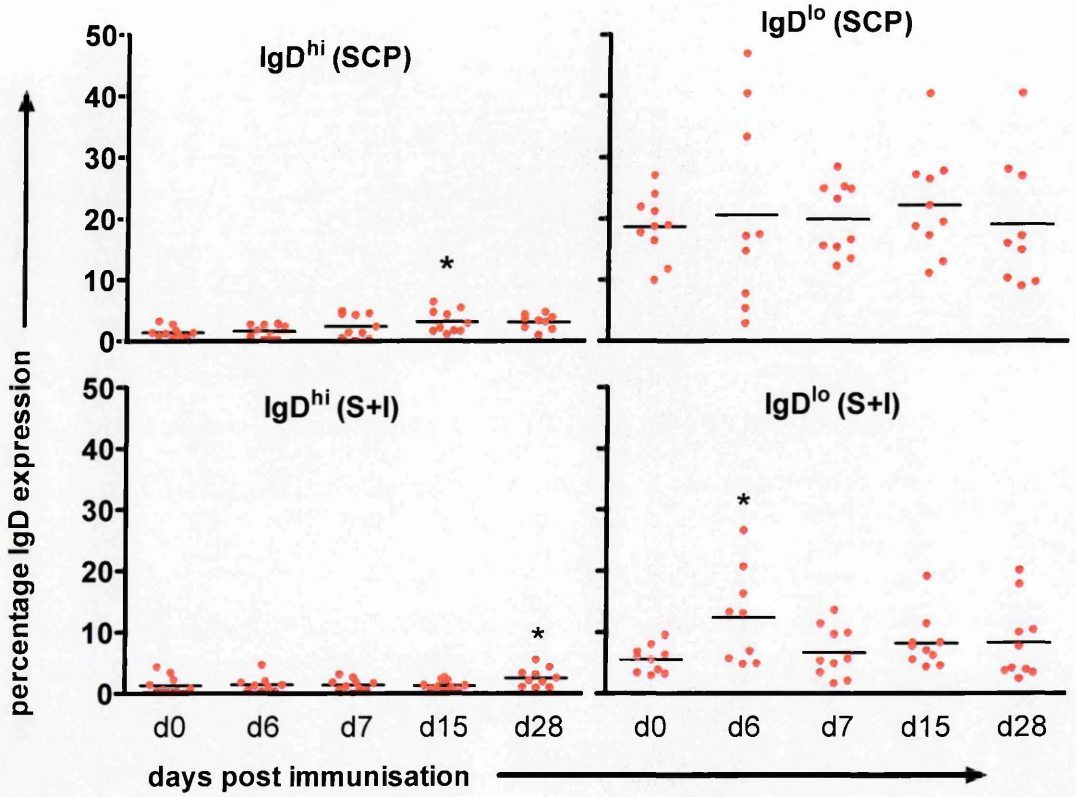


Figure 5.10 The percentage of IgD$^+$ (lo/hi) B cells present after *in vitro* stimulation of PBMCs prior to and following a single dose of Pnc7 vaccine.
PBMCs were isolated from healthy adults on days 0, 6, 7, 15 and 28 after immunisation. The cells were then stimulated for 5 days in the presence of SAC+CpG+PWM (SCP, a+b) or SAC+IL-2 (SI, c+d). On day 5 the cells were harvested and labelled for expression of surface IgD expression which was then analysed by flow cytometry. The data represent the percentage of IgD^{lo} or IgD^{hi} B cells (hi or lo fluorescence intensity) in each individual and the bar shows the mean expression in ten individuals. (*) p=<0.05 by student paired T-Test.

5.5.6 Adult B cell proliferation following immunisation Pnc7: A comparison of SCP and SI for *in vitro* stimulation.

By plotting the number of cell divisions, obtained from the CFSE dye dilution assay, against the percentage of selected B cells subsets it was possible to see the rate of proliferation following 5 day of *in vitro* stimulation. This method allowed the direct comparison between SI and SCP

stimulation. The desired outcome was to drive as many B cells as possible through at least 4 cell divisions. Figure 5.11a-p shows the kinetics of B cell proliferation after PBMCs were isolated and cultured for 5 days.

In all cases it can be seen that there were more B cells undergoing ≥4 cell divisions in the SCP system than in the SI system. However, there were no significant differences in proliferation rates between days in either system when looked at in this way.

The data were also plotted to show only the cells that had undergone ≥4 divisions (fig.5.12a-g). Slight differences between days become more apparent when the data was analysed in this way. There was a significant increase above baseline in the proliferation of $CD19^+CD27^+$ B cells in the SI system by days 7 to 15 ($p=<0.05$, fig.5.12a), while the proliferation of memory B cells in the SCP system remained constant at all time points. There was a similar trend in the naïve ($CD19^+CD27^-$) population (fig.5.12b). There were also some significant changes in the percentage of IgG^+ B cells proliferating in the SCP system with a decrease in numbers at day 6 ($p=<0.05$, fig.5.12c), and a rise in the SI system at day 7 ($p=<0.05$). The proliferation of naïve IgD^{hi} B cells appeared to peak at day 15 in the SCP system ($p=<0.01$, fig.5.12g) while no such change was observed in the SI system.

In figure 5.11, the differences between the two culture systems can be observed. In the SCP system, naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells proliferated well, although the rate appeared slower in the naïve population (fig.5.11a) with a peak in the number of cells proliferating at 4 cell divisions while memory B cells peaked at >4 cell divisions (fig.5.11c). The SI system induced no significant proliferation of naïve B cells, with a cell division number peaking at <4 divisions (fig.5.11d). Memory cells proliferated in the SI system but the percentage of these cells was much lower than in the SCP system.

There was no real difference in the proliferation of the double positive IgD^+IgM^+, IgM^+ only or IgD^{hi} B cells in the SI system (fig.5.11h, j+n), while there were more IgG^+ and IgD^{lo} B cells proliferating (fig.5.11f+l).

In the SCP system all B cell subsets proliferated but those with a more memory like phenotype, i.e. IgG^+ or IgD^{lo} (fig.5.11e+k) responded more rapidly than did the B cells with a more naïve phenotype IgD^+IgM^+, IgD^{hi} (fig.5.11h+m).

5.5.7 The predominant B cell subset proliferating prior to and following Pnc7 immunisation (SCP versus SI).

A comparison of particular B cell subsets proliferating in each culture system at each time point, prior to and following immunisation was made based on ≥4 cell divisions.

Firstly naïve and memory B cell proliferation was compared at each time point (fig.5.13). At day 0 there were significantly more memory than naïve B cells proliferating in the SI system ($p=0.011$), while there was no such difference in the SCP system. The increased proliferation of memory B cells only appeared in the SCP system at days 7 to 15 ($p=0.013$ and 0.0051 respectively), and then was lost by day 28. In the SI system, the difference between memory and naïve cells was lost following immunisation until day 15 ($p=<0.05$).

There was a slight difference in the proliferation of IgG^+ (bright green) B cell and IgM^+IgD^+ B cells (blue) in the SCP system with more IgG^+ cells making ≥4 divisions than IgD^+IgM^+ B cells ($p=0.045$). There was no difference between these two populations in the SI system at any time point.

There were significantly fewer IgD^{hi} B cells (orange) proliferating than IgD^{lo} B cells (yellow) at all time points except day 6 in the SCP system although this was most noticeable at day 0 ($p=0.0007$) and day 28 ($p=0.0001$.) The same result was seen in the SI system also.

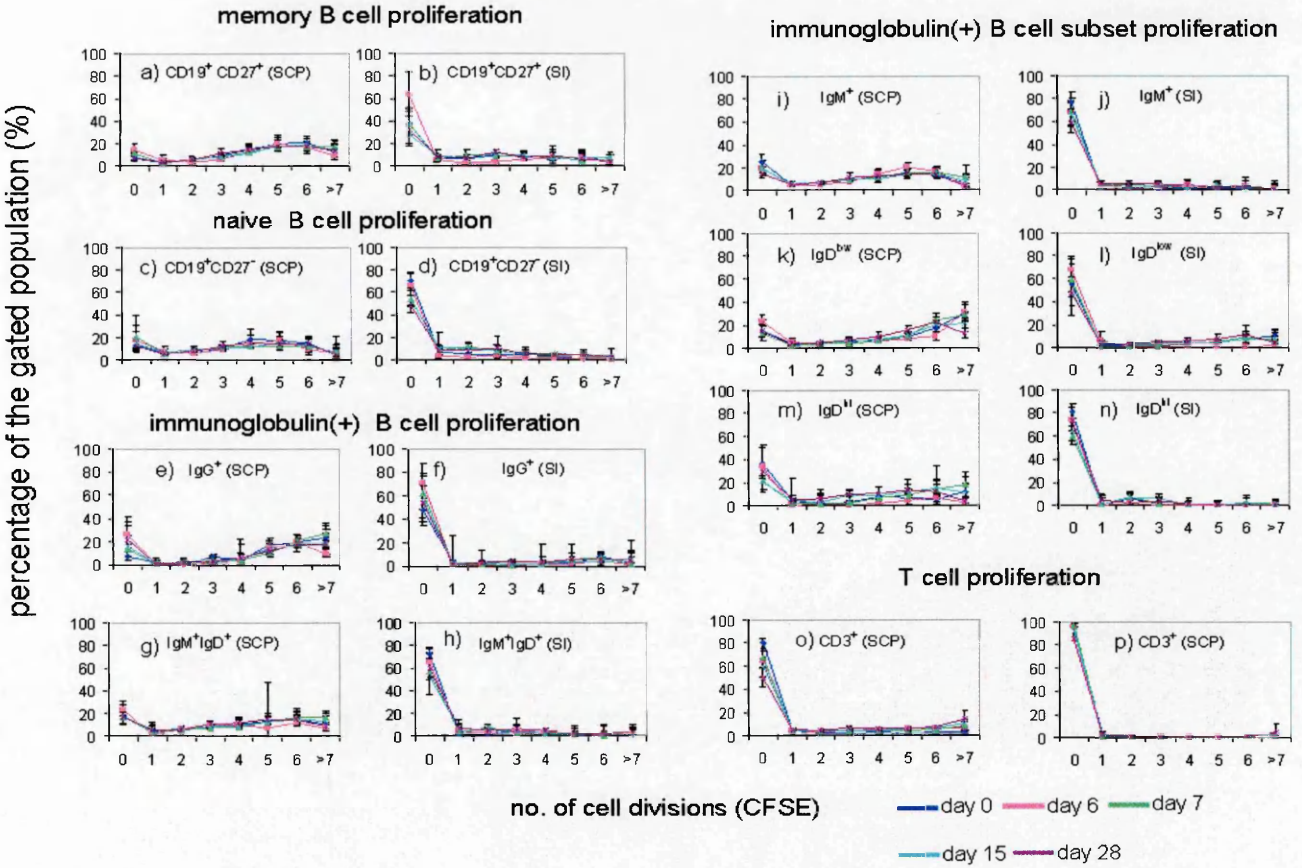


Figure 5.11 The kinetics of B cell subset proliferation.
PBMCs were isolated from adult peripheral blood on days 0, 6, 7, 15 and 28 following a single dose of Pnc7. PBMCs were labelled with CFSE and stimulated with SCP, or SI for 5 days. The PBMCs were harvested and labelled for the expression of CD19, CD27, IgG, IgD, IgM or CD3. Proliferation was then determined by CFSE dye dilution (no. cell divisions, x-axis). The data are expressed as the percentage expression of the positive populations (y-axis) per cell division (a-p). Each line represents the mean of 10 individuals with the standard error at each time point.

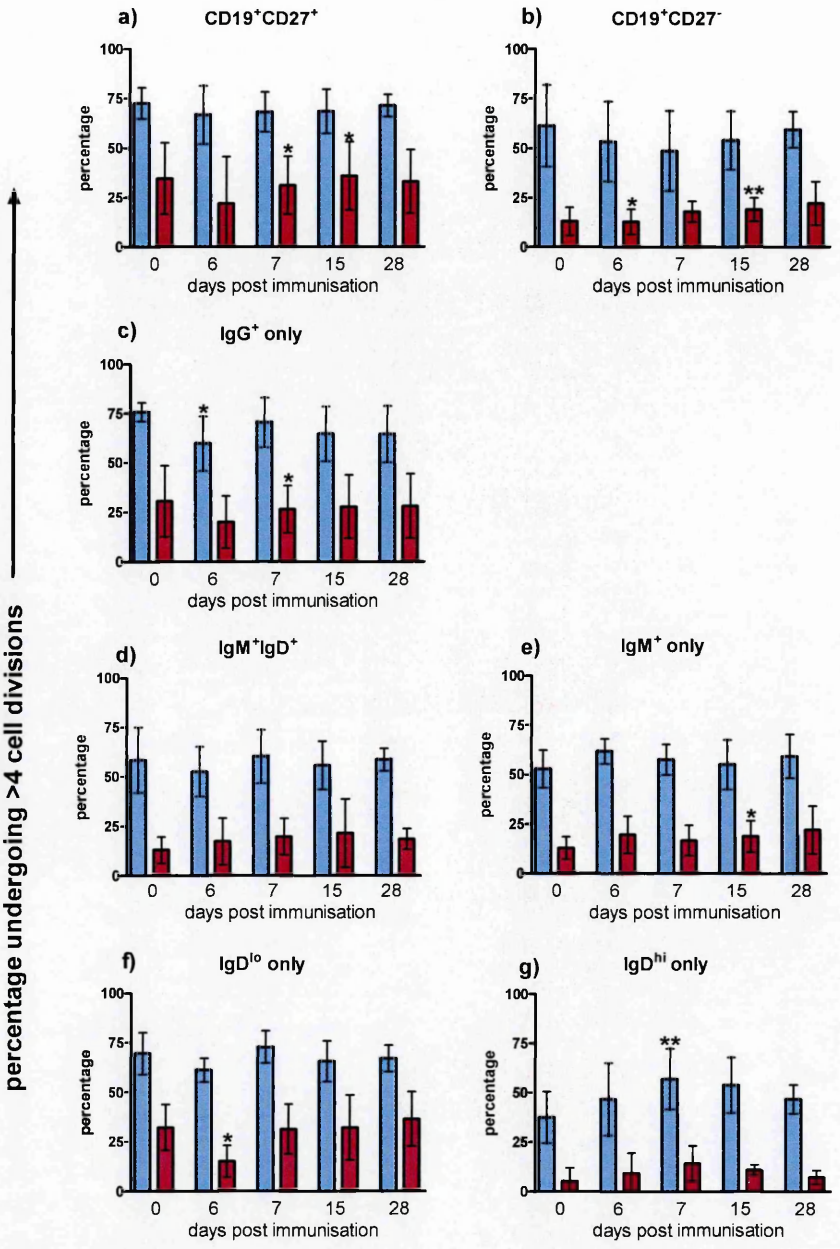


Figure 5.12 A comparison of the B cell proliferation induced by *in vitro* stimulation of PBMCs with SCP or SI.

PBMCs were harvested on days 0, 6, 7, 15 and 28 following immunisation and labelled with CFSE. The cells were then stimulated with SCP (blue) or SI (red) for 5 days. At the end of 5 days the cells were harvested and labelled for the expression of a)$CD19^+CD27^+$(memory B cells), b) $CD19^+CD27^-$ (naïve B cells), c) IgG^+ B cells, d) IgM^+IgD^+ B cells, e) IgM^+ only B cells, f) IgD^{lo} B cells and g) IgD^{hi} B cells. The percentage of these populations that had achieved >4 cell divisions (as measured by CFSE analysis) were then quantified and compared between the two culture systems. The columns represent the mean of ten individuals and the bars show the 95% CI. *p=<0.05, **p=<0.01.

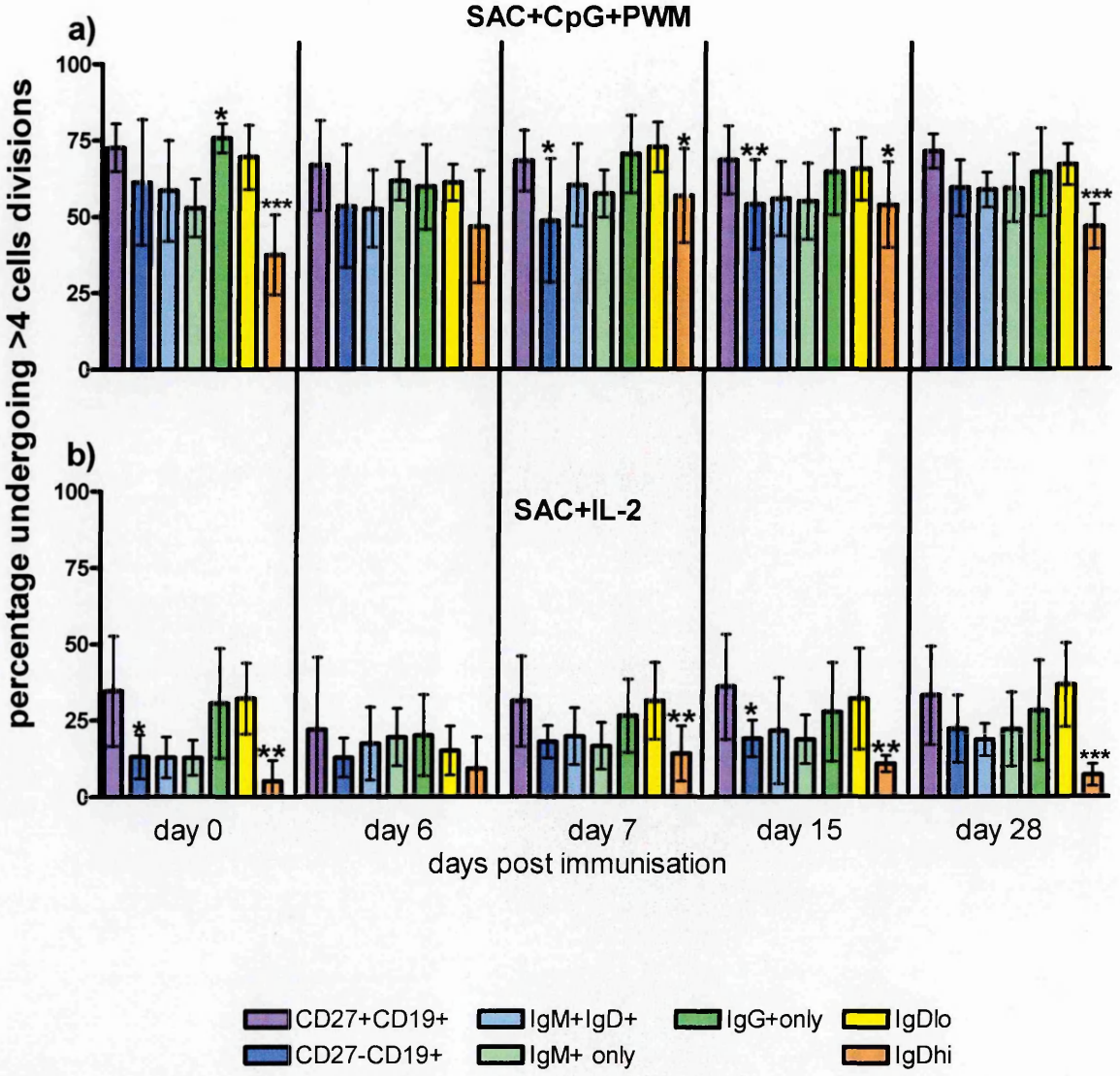


Figure 5.13 Proliferation B cell subsets following immunisation of adults with a single dose of Pnc7. PBMCs were harvested on days 0, 6, 7, 15 and 28 following immunisation, and then labelled with CFSE.

The cells were then stimulated *in vitro* with a) SCP or b) SI for 5 days. The cells were then harvested and labelled for the expression of $CD19^{+}CD27^{+}$ memory B cells (purple), $CD19^{+}CD27^{-}$naïve B cells (dark blue), $IgM^{+}IgD^{+}$ B cells (light blue), IgM^{+} only B cells (pale green), IgG^{+} B cells (bright green), IgD^{lo} B cells (yellow) and IgD^{hi} B cells (orange). The percentage of these populations that had achieved >4 cell divisions (as measured by CFSE analysis), were then quantified by flow cytometry and compared between the two culture systems. The columns represent the mean of ten individuals with 95%CI. *p=<0.05, **p=<0.01, ***p=<0.0008.

5.5.8 Depletion or enrichment of peripheral blood B cells expressing IgD^+ or IgG^+ and the subsequent proliferation of these B cells *in vitro*.

PBMCs were isolated from two adult donors (donor 1 and donor 2, fig.5.15) immunised with Pnc7, more than 6 months previously, were labelled with CFSE to allow determination of the proliferation of B cell subsets. The proliferation of the cells within these fractions was analysed by CFSE dye dilution, an example analysis of which is shown in figure 5.14. The percentage of cells undergoing art least 1 cell division was quantified (marker M2 fig. 5.14).

From the donor 1 experiment (fig.5.15) it can be seen that depletion of either IgG or IgD led to decreased proliferation of the IgG^+ and IgD^+ B cells when compared to the un separated fraction. The same effect was seen within the total $CD19^+$ populations. The proliferation of IgM only memory cells ($CD27^+IgM^+$) was unaffected by the depletion of IgG or IgD B cells and compared well to the un separated fraction. The proliferation of the naïve B cells ($CD27^-IgM^+$) was affected more by the depletion of IgD than IgG, but both depletions dramatically reduced the amount of proliferation seen in comparison to the unseparated cells.

In the donor 2 experiment (fig.5.15), where the depleted and enriched fractions were compared directly the following observations were made. Firstly there was an equivalent percentage of surface IgG^+ B cells proliferating in the IgG^+(dark green) and IgD^+(purple) enriched fractions and in both cases the enriched fractions proliferated more than the depleted (IgD^-, IgG^- fractions). There was a higher percentage of IgD^+ B cells proliferating in the IgD enriched fraction than in the IgG depleted fraction while no IgD^+ B cells were seen proliferating in the IgG^+ enriched or IgD depleted fraction. There was a higher percentage of total $CD19^+$ B cells proliferating in the IgD enriched fraction than in either the IgG enriched or depleted fraction. There was no proliferation in the IgD depleted fraction. Proliferation of $CD27^+IgM^+$ B cells was considerably higher in the IgD enriched fraction. Enrichment or depletion of IgG^+ B cells had no effect on the proliferation of $CD27^+IgM^+$ cells. Naïve B cell proliferation was enriched in the IgD^+ enriched

fraction and there was also some proliferation of naïve cells in the IgG depleted fraction but this was lower than the IgD enriched fraction.

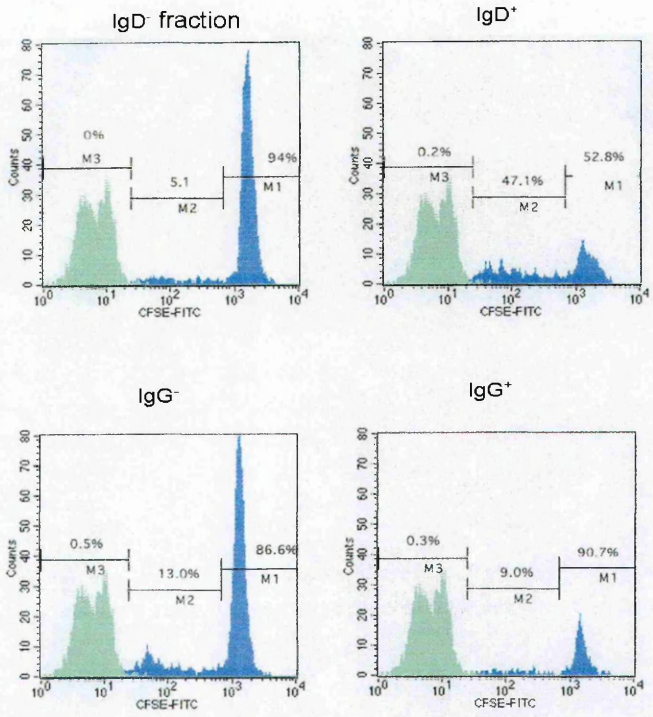


Figure 5.14 B cell subset proliferation as measured by CFSE dye dilution.
PBMCs from a healthy adult donor were labelled with CFSE, a fraction were kept unlabelled as the negative cut off for cell division numbers, shown by marker (M) 3, (green). The PBMCs were then separated by AutoMACs™ on the basis of IgD or IgG expression. The separated cells were then cultured for 5 days in the presence of SAC+CpG+PWM. Following this the cells were harvested the percentage of B cells proliferating (blue) in the IgD+/- and IgG+/- fractions was quantified by flow cytometery on the basis of CFSE dye dilution (x-axis). M1 shows the non-proliferating cells and M2 the percentage of B cells that proliferated in each fraction.

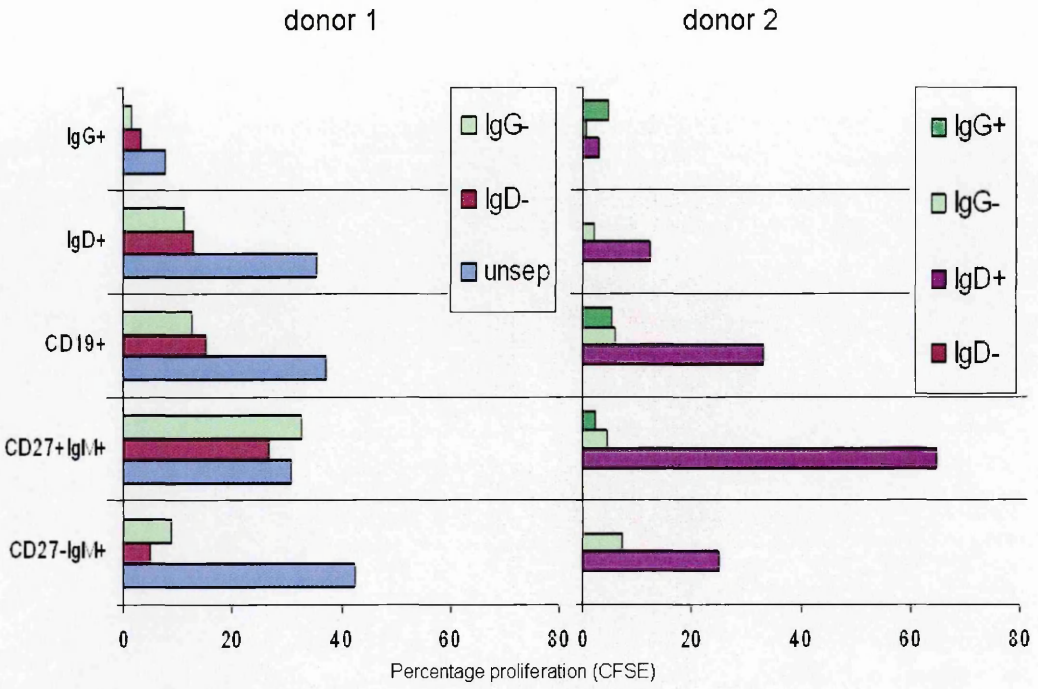


Figure 5.15 Proliferation of PBMCs depleted or enriched for IgD+ or IgG+ B cells.
PBMCs were isolated from two adult donors (donor 1 and donor 2), who had previously been immunised with Pnc7. The PBMCs were labelled with CFSE as described previously and were then separated on the basis of either IgG or IgD expression. Each of the fractions, along with an unseparated PBMC control, were stimulated for 5 days in the presence of SAC+CpG+PWM. At the end of the 5 day stimulation culture the cells were harvested and labelled for IgG, IgD, CD19, $CD27^{+}IgM^{+}$ and $CD27^{-}IgM^{+}$ expression which was anaylsed by flow cytometry. The proliferation of each of these subpopulations was expressed as the percentage of cells that had undergone at least 1 cell division as determined by CFSE dye dilution as shown in figure 5.14.

5.5.9 The purity of the depleted and enriched PBMC fractions following IgD based cell sorting

In order to determine the purity and phenotype of the cells within each of the fractions obtained by AutoMACs™ cell sorting, the percentage of lymphocyte populations was determined (figure 5.16). Data was collected from five individuals and the percentage surface expression of CD27, CD19, CD3, IgM, IgD (5.16a) and subpopulations based on the percentage of IgD and IgM

expressed within the $CD27^+$ or $CD27^-$ populations (5.16b) within each of the sorted fractions were analysed.

The overall B cell population ($CD19^+$) was enriched by sorting on IgD expression. The percentage of $CD19^+$ B cells in the un-separated PBMCs was 12% and this was enriched to 64% in the IgD^+ sorted fraction (fig.5.16a). The percentage of IgD^+ B cells was enriched by the sorting process so that in the un-separated PBMCs there was 11% IgD^+ B cells while in the sorted IgD^+ fraction the proportion of IgD^+ B cells increased to 62%. There was a concurrent enrichment of IgM^+ B cells going from 9% in the un-separated fraction to 59% in the IgD^+ sorted fraction. There was less than 1% IgD^+ or IgM^+ B cell in the IgD^- sorted fraction, yet there remained 2.85% $CD19^+$ B cells that may have been the IgG^+ B cells.

The naïve ($CD27^-$) lymphocyte population was enriched in the IgD^+ fraction compared to the IgD^- fraction (62% vs 23%), while the reverse was true for the $CD27^+$ population (37.9% vs 77%), although this would include $CD27^+$ T cells as well as B cells.

The B cell subsets were also different between the IgD^+ and IgD^- fractions (fig.5.15b). There was less than 0.5% IgM only B cells in either the $CD27^+$ or $CD27^-$ populations in any of the fractions. The $CD27^-IgM+IgD^+$ population was enriched in the IgD^+ fraction with 72% versus 18% and 0.1% in the un-separated and IgD^- fractions respectively. The $CD27^+IgM^+IgD^+$ population was similarly enriched although was much lower than the $CD27^-$ population (21%, 1.7% and 0.2% in the IgD^+, un-separated and IgD^- fractions respectively). There was similar enrichment of the IgD^+only B cell population in the sorted versus un-separated fractions.

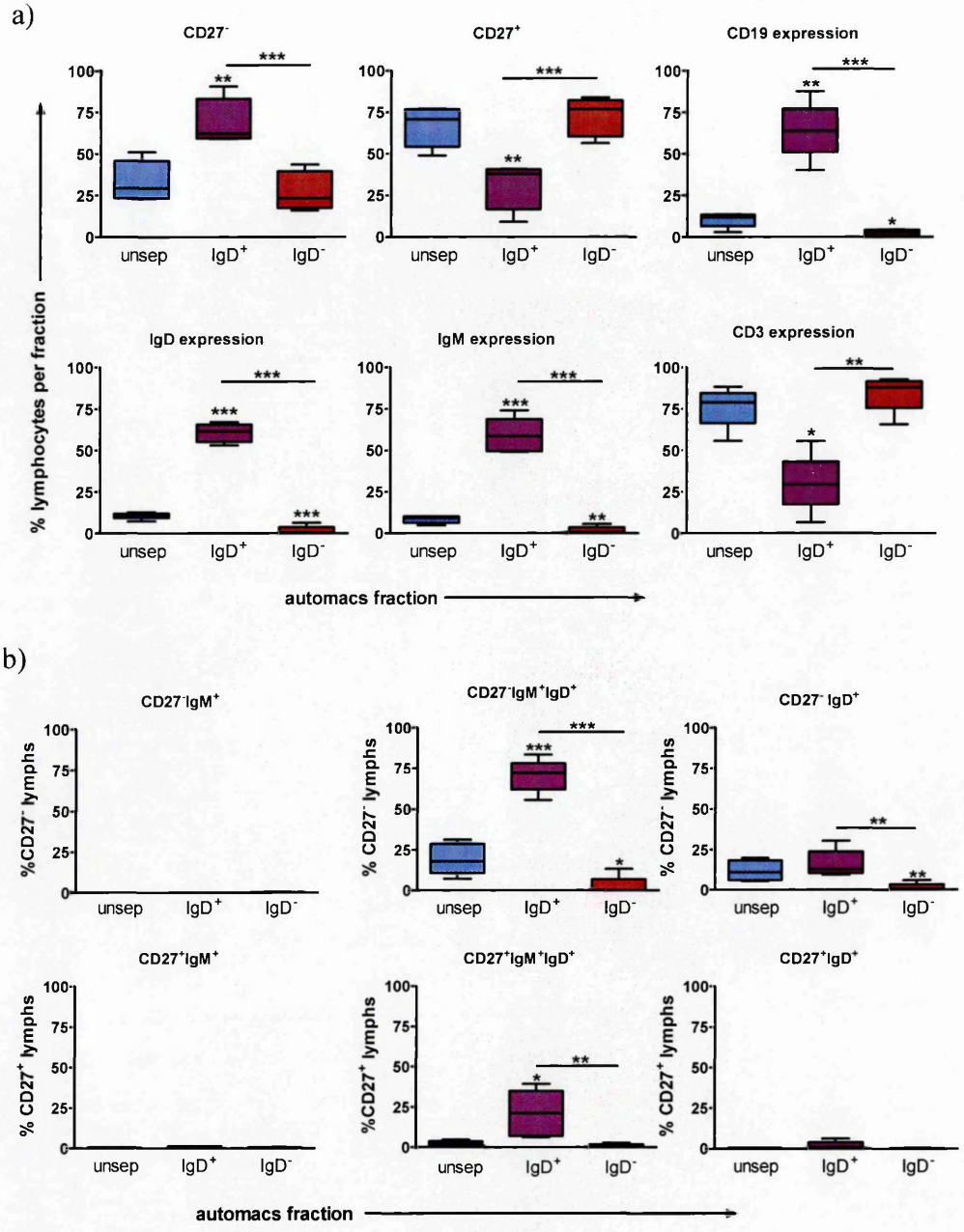


Figure 5.16 The purity of the magnetic cell sorting based on IgD expression.
PBMCs were sorted on the basis of IgD expression and then the total lymphocyte subsets within each fraction were determined. a) $CD27^-$, $CD27^+$, $CD19^+$, IgD^+, IgM^+ and $CD3^+$ populations and b) show the naïve ($CD27^-$) B cell populations in the top row $CD27^-$ IgM^+, $CD27^-$ IgM^+IgD^+ and $CD27^-$ IgD^+ and the memory ($CD27^+$) B cell populations on the bottom row $CD27^+IgM^+$, $CD27^+IgM^+IgD^+$ and $CD27^+IgD^+$. The data represent the median, 25^{th} and 75^{th} percentiles with outliers for five individuals.

5.6 Discussion

The data presented in this chapter show that the nature of the polyclonal stimulation given to peripheral blood B cells affects the type of polysaccharide specific IgG-AFC detected by ELISpot following recent immunisation with a glycoconjugate vaccine. No such effect was observed in relation to the carrier protein specific IgG-AFC (diphtheria toxoid). The frequency of total IgG-AFC was significantly higher following B cell stimulation with SCP compared to SI and this was also true of IgG-AFC specific for the non-vaccine related protein, tetanus toxoid.

5.6.1 SAC+CpG+PWM stimulation of PBMCs generates higher frequencies of IgG-AFC than is seen with SAC+IL-2.

The frequency of total IgG-AFC harvested from SCP cultures compared to SI cultures was always higher, but remained at a constant frequency during the time course. This was also the case for tetanus toxoid specific IgG-AFC. Although the frequency of diphtheria toxoid specific IgG-AFC increased following immunisation there was no significant difference between the SCP and SI systems, except that the peak days were different. Therefore, detection of IgG-AFC specific for a recently administered protein antigen did not appear to be influenced by the system of polyclonal, *in vitro* B cell stimulation used.

There did appear to be differences in the activation requirements of polysaccharide specific B cells that became apparent following polyclonal stimulation with either SCP or SI. At baseline and also on days 15 and 28 following immunisation the *in vitro* stimulation of PBMCs with SCP induced higher IgG-AFC frequencies specific for pneumococcal capsular polysaccharides. This result suggests that the polysaccharide specific B cells, present in the peripheral blood prior to and 2-4 weeks after immunisation have different activation requirements than those seen 6/7 days after immunisation.

In a previous study, PWM stimulation of PBMCs generated increased frequencies of anti-pneumococcal polysaccharide specific AFC from two weeks after immunisation (434), which supports the findings in this study with SAC+CpG+PWM, where the peak in antigen specific AFC was seen at day 15 verses day 7 with SAC+IL-2. Thus, T cell dependent polyclonal activation of B cells is more efficient from 15 days post immunisation, while T cell independent B cell stimulation works more efficiently during the first two weeks after immunisation. This suggests an intrinsic difference in the co-stimulatory requirements of the B cells present in peripheral blood during these two time periods. To further support this hypothesis, a study from 1983 of *in vitro* antibody formation following pneumococcal polysaccharide vaccine in humans (109), demonstrated a suppressive effect of PWM (T cell dependent, polyclonal stimulation), on early antibody responses while the *in vitro* response of B cells to specific polysaccharide antigens remained intact during the first week after immunisation. In contrast at two weeks after immunisation the reverse was true with *in vitro* responses to specific polysaccharide antigens disappearing and polyclonal stimulation by PWM leading to polysaccharide specific antibody secretion *in vitro*.

The data in this chapter study, therefore, supports the role of SAC+IL-2 as a T cell independent antigen, behaving in a manner similar to specific polysaccharide antigen. *In vitro* stimulation of PBMCs with SAC+IL-2, promotes the differentiation of activated B cells into AFC during the first week after immunisation. This effect returns to baseline by day 15-28 when such B cells have migrated to bone marrow or returned to lymphoid tissues. The similar frequencies of IgG-AFC seen following SAC+CpG+PWM stimulation at this stage in the vaccine response suggests that PWM may be antagonising the combined effects of SAC and CpG. Thus only the same subset of B cells will differentiate into AFC. By day 15 this effect disappears and much higher AFC frequencies are induced by SAC+CpG+PWM than by SAC+IL-2. A similar suggestion was made following studies of human anti-sheep red blood cell (SRBC)-AFC induced by either SAC

or PWM (461). Here it was shown that specific antigen (SRBC) was required to stimulate human B cells before a SAC or PWM induced response was observed.

The data from this study combined with these previous observations suggests a role for PWM activated T cells in the generation of AFC and that they may have a suppressive effect during the first week to 14 days after immunisation.

5.6.2 Stimulation of PBMCs with SAC+IL-2 induces memory B cell differentiation.

The literature suggests that the use of SAC alone induces polyclonal activation of B cells in a T cell independent manner, and seems to exert its effect on B cells that have recently been exposed to their specific antigen (437, 438). There is conflicting evidence about whether SAC alone is able to induce full differentiation of B cells into IgG-AFC (437, 444, 462), or not and whether the addition of IL-2 provides an extra stimulus (438, 444, 463, 464).

A two signal hypothesis was suggested where, signal 1 was provided via surface Ig cross-linking (437, 440, 444), and signal 2 was provided by concurrent exposure to T cell factors such as IL-2 (438, 465). B cell receptor cross-linking by antigens such as SAC leads to up-regulation for the IL-2 receptor alpha (IL-2R-α) chain (or CD25), on the B cell surface enhancing proliferation of the activated B cell. It was later shown that IL-2 enhanced the expression of Bcl-X_L (a survival signal related to Bcl), in B cells activated by SAC mediated BCR cross-linking that enhanced the survival of these cells in culture (439, 443). However, the combination of SAC with IL-2 still only activated memory B cells to differentiate into IgG secreting cells (376, 377, 444).

The ELIspot data presented in this chapter suggests that SAC+IL-2 exerts an effect on different B cell subset to that seen with SAC+CpG+PWM because there is a peak in AFC formation at day 7 verses day 15. In a study be Suzuki *et al* (444), it was observed that high density human B cells stimulated with SAC alone (in the absence of T cell factors) proliferated but did not differentiate in to antibody secreting cells, while low density B cells did differentiate into antibody secreting

cells without the need for further *in vitro* proliferation. This may be what is occurring in the first week after immunisation.

5.6.3 *In vitro* co-stimulatory signals provided *in vitro* directly affect the AFC frequency detected in the ELISpot assay

In the week following immunisation antigen specific B cells were proliferating *in vivo*. They were then placed into the *in vitro* culture system using SAC+IL-2, which induced antigen activated B cells to differentiate into antibody secreting cells, the frequency of which peaked at days 6/7 after immunisation but had returned to baseline 1 month later.

In the case of SAC+CpG+PWM the scope of B cell activation is enhanced by the provision of three separate signals for B cell activation. SAC provides the BCR signalling while PWM provides direct B cell stimulation but requires T cell help to do so (442, 466). Rapid build up of T cell factors in PWM stimulated cultures, which can then be used to provide help to the B cells was demonstrated in work by Dosch *et al*-1980 (437). It has also been shown that PWM activates the early growth response gene (EGR)-1 in B cells, but that the effect of PWM is concentration dependent (467). Saiki *et al* (464), then showed that low concentrations of PWM worked in synergy with SAC to provide optimal B cell activation and differentiation into antibody secreting cells.

There is only a small subset (<1%) of peripheral blood B cells that respond to PWM and it has previously been shown in humans that they are surface IgG positive but produce IgM antibody (468, 469). So the combination of SAC+PWM satisfies similar signalling requirements to those provided by SAC+IL-2. It would still appear that this system might only enhance the proliferation and differentiation of memory B cells however. So for the difference in the ELISpot data obtained from the two systems attention must turn to the effects of CpG-ODN-2006 on B cell activation.

CpG-ODN-2006 activates both naïve and memory B cells in a polyclonal manner but the requirements for the activation of memory versus naïve B cells differ (228, 470, 471). CpG-DNA activates B cells via interaction with TLR-9 which is expressed in intracellular vesicles (455). CpG-DNA may be taken up by pinocytosis or endocytosis and then co-localises with TLR-9 in endosomal vesicles (472), as TLR-9 is not expressed at the surface unlike TLR-4 and 10 (457). Cross-linking of the BCR leads to up-regulated expression of TLR-9 in naïve B cells (228, 351, 471). The interaction of CpG with TLR-9 leads to up-regulation of co-stimulatory molecules such as CD80, MHC class II and CD40 on the B cell surface and enhanced antigen presenting capabilities of the naïve B cell (449), so that it is then able to recruit antigen specific T cell help which provides the signals for B cell differentiation into antibody secreting cells. What this means for the ELISpot outcome is that peripheral blood B cells isolated prior to and following immunisation will be activated by this combination of polyclonal stimulants irrespective of how recently they were exposed to their specific antigen. The total IgG-AFC frequency was much higher because SCP is able to induce proliferation and differentiation of naïve, resting memory and activated memory B cells while SAC+IL-2 only induces differentiation of antigen activated memory B cells. During the week after immunisation SCP and SI induce equivalent numbers of IgG-AFC. This suggests that the ability of the B cells present in the circulation at this time to proliferate and differentiate into plasma cells is independent of *in vitro* T cell help or TLR-9 signalling. Diphtheria specific B cells appear to proliferate and differentiate independently of T cell and TLR-9 signalling throughout the study time course while it is restricted to days 6/7 for the polysaccharide specific B cells.

By day 15 to 28 the B cells present in the circulation appear to be a mixture of cells that require signalling via TLR-9, BCR and T cell help for differentiation and those that require only SAC+IL-2.

Therefore the data in this chapter show that there does appear to be a distinct difference in the activation requirements of polysaccharide specific B cells depending on the time at which they

are isolated following immunisation. In order to determine the effect of the different polyclonal stimuli on PBMCs CFSE labelling was used to look for differences in proliferation lymphocytes stimulated with the individual or combined mitogens.

5.6.4 CFSE analysis of lymphocytes reveals differences in the effects of SAC, PWM, CpG on B cell proliferation.

Using PBMCs isolated from an un-immunised individual it was possible to look at the proliferation of the B cells in response to the polyclonal stimuli. Combining CpG with either PWM or SAC appeared to synergistically enhance lymphocyte proliferation compared to either PWM or SAC alone. Rush *et al*-2002 showed that B cells could be stimulated with CpG and T cell-B cell interaction via CD40-CD40L and Jiang *et al*-2007 showed that CpG could stimulate the up-regulation of CD40 on the B cell surface in the absence of BCR cross-linking. SAC and CpG augment each other since SAC interaction with the BCR leads to TLR-9 up-regulation and CpG-TLR-9 interaction lowers the activation threshold of the B cells (455, 473). Thus, combining SAC+CpG+PWM resulted in the maximal proliferation of both $CD27^-$ and $CD27^+$ B cells. This is important for the outcome of the ELISpot since differentiation, immunoglobulin secretion and class switching all rely on B cell division number (387, 416, 417, 451, 474). Secretion of antibody requires proliferating B cells to have undergone at least 4 divisions (the M5 marker in figure 5.4), (387, 416, 417, 474).

The level of proliferation correlated with the outcome of the ELISpot shown in figures 5.8 and 5.9, in that cultures with the highest degree of proliferation resulted in more IgG-AFC detected in the ELISpot.

Individually SAC, PWM and CpG all induced lymphocyte proliferation in the CFSE analysis and IgG secretion in the ELISpot assay. However, combining all three dramatically increased the amount of proliferation and also antibody secretion (IgG and IgM, figure 5.8), both to the polysaccharide antigens and protein antigens compared to the individual stimulants. There was

also a much higher frequency of IgM-AFC produced than IgG-AFC and this appeared to be fairly non-antigen specific.

Individually SAC (418, 462), CpG (470, 475) and PWM (468, 469) induce IgM production as well as IgG and the CFSE proliferation data in figure 5.6 shows this clearly. CpG elicited an IgM^+ B cell response from both naïve and memory B cells, and a previous study (470), showed that the IgM antibody was secreted from naïve B cells while the IgG was from memory B cells in response to CpG. The difference in isotype may be related to rapidity of proliferation of $CD27^+$ versus $CD27^-$ B cells since a higher divison number is required for IgG secretion then IgM (387, 416, 417, 474).

SAC and PWM only induced proliferation of memory B cells as already discussed. Combining CpG with SAC or PWM or both lead to progressive down regulation of IgM which suggests that class switching was occurring with this combination of antigens while CpG alone appeared to maintain IgM^{hi} B cells.

Having established from these data that there were obvious differences in the types of B cell being maintained in cultures using these different mitogens the next step was to determine whether it was possible to detect gross differences in B cell subset proliferation at different time points after immunisation.

5.6.5 Proliferation of IgD^+ B cells is induced by *in vitro* stimulation with SCP and SI.

Only proliferation of IgD expressing B cells appeared to be altered following immunisation, with a rise in the percentage of proliferating IgD^+ cells by day 7 in response to SCP stimulation peaking at day 15. This effect was delayed in the SAC+IL-2 system. Saiki *et al*-1982 showed that removal of IgD^+ B cells led to decreased IgM-AFC responses following SAC and PWM stimulation while the removal of IgM^+ B cells lead to a complete loss of the IgM-AFC response. The IgG-AFC response to SAC and PWM was unaffected by depletion of these cells.

The limited cell sorting data shown in this chapter suggests that magnetic cell sorting to deplete IgD^+ cells preferentially removes the IgD^{hi} B cell population because, a) there were still some IgD^+ B cells in the IgD negative fraction and b) there was a reduction in the naïve ($CD27^-IgM^+$) B cell proliferation in the IgD depleted fraction compared to the unseparated PBMCs. Also there was no difference in the proliferation of un-switched $CD27^+IgM^+$ memory B cells (mostly IgD^{lo}), compared to the un-separated PBMCs or IgG depleted fractions (fig.5.15). This preferential removal of IgD^{hi} naïve cells could explain why the SAC and PWM induced IgM response was reduced while the IgG response remained. Another murine study by Defrance *et al*-1992 showed that IgD^+ cells mediated the IgM response while IgG^+ B cells secreted IgG, IgM and IgA. Since the proliferation of IgD expressing cells was altered following immunisation, the overall percentage of IgD expressing B cells remaining in culture after 5 days stimulation was determined (figure 5.9).

IgD^{hi} B cells are classically naïve ($CD27^-IgM^+IgD^{hi}$) while IgD^{lo} B cells are memory ($CD27^+IgM^+IgD^+$) B cells (124, 320, 321). It is the $CD27^+IgM^+IgD^{lo}$ B cells that are implicated in polysaccharide responses (252, 267, 321) and the data in this chapter show a relationship in the timing between IgD expressing B cell frequency and IgG-AFC frequency (fig.5.2. and 5.9 and 5.10).

There is a peak in IgD^{hi} B cells that coincides with the peak in IgG-AFC seen in the ELISpot assay following stimulation of PBMC with SAC+CpG+PWM, while the frequency of IgD^{lo} (memory) B cells remained constant through out the time course. As has been discussed already, this combination of antigens stimulates memory and naïve B cells. There was a peak in IgD^{lo} (memory) B cell frequency in the SAC+IL-2 system that peaked on day 6 after immunisation. The peak in IgG-AFC was at day 7 in this system so frequency of memory B cells did not directly tie with IgG-AFC frequency in this system. However, one suggestion might be down regulation of IgD as the B cells class switched. This is hard to confirm without looking at the kinetics of B cell subset proliferation. (See figure 5.11 and fig. 5.12).

5.6.6 *In vitro* stimulation, but not immunisation, affected B cell subset proliferation kinetics

The memory B cell subsets ($CD27^{+}CD19^{+}$, IgG^{+}, and IgD^{lo},) all underwent more rapid proliferation than the naïve B cells, with the majority achieving ≥4 cell divisions. This was more apparent in the SCP system than in the SI system. Also apparent was the difference in the effects of the two systems on the proliferation of naïve B cells. Very few achieved the target ≥4 cell divisions in the SI system which is of no great surprise since we now know that this combination of antigens only induced proliferation of *in vivo* activated B cells. There was an accumulation of B cells from all subsets in the SCP system after 5 days of culture and all achieved a high percentage of B cells undergoing ≥4 cell divisions. The high percentage of B cells achieving >4 cell divisions in the SCP system may be the reason for the higher frequencies of IgG-AFC detected in the ELISpot compared to that seen with SI stimulation. Experiments carried out in mice to look at *in vitro* induced class switching revealed results that are similar to those shown in figure 5.11. Hodgkin-1996 and Hasbold-1998 stimulated small, dense, resting murine B cells with plasma membranes from Th cells plus T cell supernatants. In both of these studies there was an observed increase in the expression of IgG after cultured B cells had undergone at least 2 cells divisions. The data reported here show that the SCP system, which is the T cell dependent system showed similar kinetics for the up-regulation of IgG while in the SI system this was delayed until 5 cell divisions. This suggests a delay in class switching in the absence of direct T cell help in the SI system. The murine studies reported loss of IgD expression in cultured B cells after 2 cell divisions with complete loss by 4 cell divisions. In this study the SCP system maintained IgD expressing B cells while the SI system induced a loss of IgD^{hi} B cell expression by 4 cell divisions. Thus it seems that naïve B cells did not survive in culture with SAC+IL-2 alone. The kinetics of IgM down regulation or class switching was similar in the murine studies and in the SCP system in this study with IgM loss following 6-7 cell divisions. In the SI system, IgM only B cell expression was negligible. There was no effect of bleed day on the kinetics of proliferation

in either system although individuals varied. Other murine studies (446, 476) have shown a relationship between division number and Ig germ line gene mRNA transcript expression and class switch recombination that is regulated by cytokines such as IL-4 (IgG1, IgE), IFNγ (IgG2a), and TGFβ (IgA), in conjuction with CD40L co-stimulation of the B cells.

In conclusion, these experiments show that PBMC stimulation with SAC+CpG+PWM versus SAC+IL-2 enables distinction to be made between recently activated B cells and resting B cells. There were also differing *in vitro* effects on the anti-polysaccharide response compared to the protein (anti-diphtheria) response.

SAC+CpG+PWM enables the detection of polysaccharide specific IgG-AFC within the peripheral blood at anytime, while SAC+IL-2 is useful for defining the actual peak in frequency of vaccine induced IgG-AFC (fig.5.17).

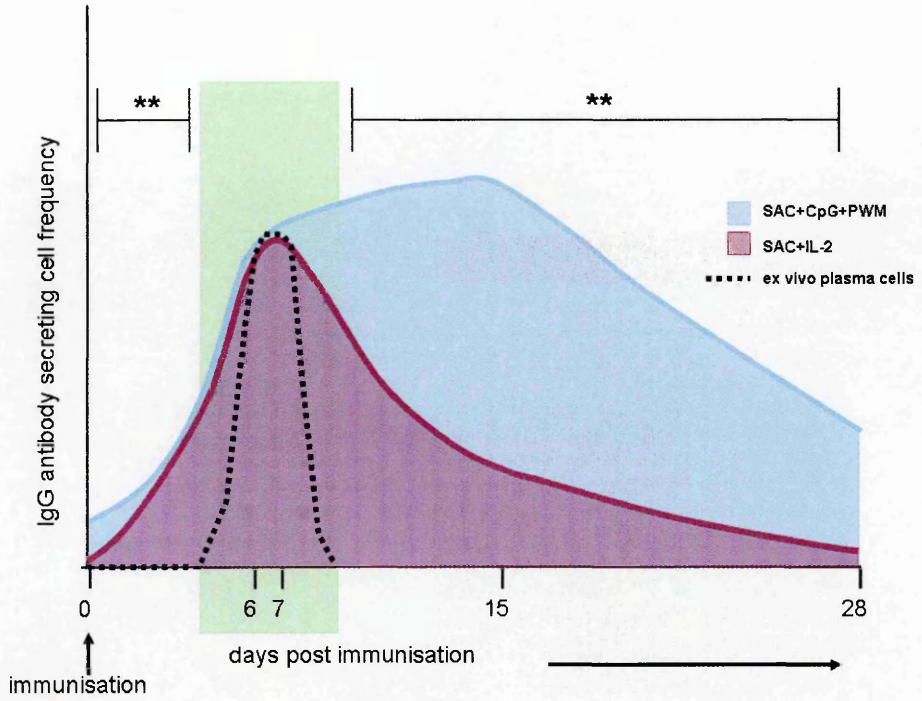


Figure 5.17 The kinetics of IgG-AFC frequency following immunisation with Pnc7.
During the first week after immunisation, there was no difference in the signalling requirements of B cells detected in either polyclonal stimulation system. At baseline (**)or from two weeks post vaccination onwards (**) there was a very low frequency of B cells that did not require T cell help or TLR-9 co-stimulation for proliferation and differentiation during *in vitro* culture. Therefore the sensitivity of the SAC+IL-2 system was reduced in un-immunised volunteers or at time points beyond seven days post immunisation. The CFSE proliferation studies show clear differences in the B cell subsets maintained during *in vitro* stimulation with SCP vs SI, with the predominant difference being maintenance of naïve B cell responses in the SCP system and bystander stimulation of $IgD^{+}IgM^{+}CD27^{+}$ B cells implicated in anti-polysaccharide responses.
Since the SAC+CpG+PWM system enabled the detection of pre-existing polysaccharide specific B cells in the ELISpot prior to immunisation this system was chosen for to look at the effect of age on the response to pneumococcal conjugate vaccine to avoid biases that may be introduced by an ageing or immature immune system.

Chapter 6: The effect of age on the development of immune memory in response to Pnc7 immunisation.

6.1 Abstract

The effects of age on the B cell response to a primary dose of Pnc7 were compared between toddlers and young adults, and elderly versus young adults. Toddlers received two doses, 2 months apart. Elderly adults were also grouped to receive a primary dose of Pnc7 or 23PsV to compare the B cell response to a TD- versus TI-2 vaccine.

The primary Pnc7 dose in toddlers induced a rise in plasma cell frequency by day 7 and this was also seen following secondary immunisation, though there was no difference in magnitude. There was also a steady rise in memory B cell frequency seen following the primary and secondary immunisation (using SAC+CpG+PWM *in vitro* stimulation).

When compared to the response in young adults the plasma cell frequency was significantly lower in the toddlers than the adults and the difference was maintained after the second dose.

The total-IgG memory B cell frequency was significantly lower in the PBMCs from the toddlers at all time points than in the adult's PBMCs. The memory response to the polysaccharide components of the vaccine was significantly lower in toddlers than in adults after primary immunisation. However, 7 days after a second dose of Pnc7 toddler memory cell frequencies equalled that of adults after a single Pnc7 dose.

When young adults were compared to elderly adults following a primary dose of conjugate vaccine there was no difference in the memory B cell response between the groups, even though at baseline, the frequencies of polysaccharide specific memory B cells were lower in the elderly. Comparison of toddlers, young adults and elderly adults at baseline revealed no difference in tetanus or diphtheria specific memory but highly significant differences in polysaccharide specific memory cell frequency (adults>elderly>toddlers).

The serum antibody response in toddlers was slower to mature than in young adults. After the primary dose of Pnc7 IgM still predominated in response to serotype 14 and 23F while serotype 4 induced a similar response in both age groups (IgG, IgA and IgM). After the second toddler dose IgG was equivalent between the age groups for serotype 14 and 23F and higher for type 4 in the toddler group.

Characterising the response of elderly adult B cells to *in vitro* stimulation with SAC+CpG+PWM and SAC+IL-2 revealed a significantly lower rate of proliferation of $IgM^{+}IgD^{+}$ B cells in both culture systems at day 0 when compared to that seen for young adults. This was also evident at day 28 in the SCP system. The elderly adults also had decreased proliferation of $CD27^{+}$ B cells at day 0 and day 28 than was observed for young adults.

When the SCP and SI systems where used to compare the response of elderly adults to a single dose of Pnc7 versus 23PsV the outcome of the response to immunisation was significantly different. *In vitro* stimulation with SCP revealed a significant increase in memory B cell frequency following conjugate immunisation but this was lost when SI was used to stimulate the B cells. In response to the polysaccharide vaccine, neither SCP nor SI showed a significant rise in memory cell frequency.

Conclusion

Toddlers require at least 2 doses of Pnc7 to generate memory responses equivalent to adults. Young and elderly adults are able to mount equivalent memory responses to Pnc7 immunisation even though there appears to be some difference in the quality of the B cell subsets induced by *in*

vitro stimulation of PBMCs. Immunisation of elderly adults with a conjugate vaccine did not induce significantly more memory B cell IgG-AFC when compared with the polysaccharide vaccine.

6.2 Introduction

The pneumococcal-CRM197 conjugate vaccine was introduced into the UK infant immunisation schedule in 2006 and involves three immunisations while un-immunized children who were in their second year of life were offered a single dose of the vaccine in a catch up campaign. However, at 12 months of age, a single dose of heptavalent conjugate vaccine may not be sufficient to induce protective antibody levels (a protective level has been variously described as >0.2mcg/ml, or 0.35μg/ml or 1.0μg/ml) (174, 362) to all seven serotypes included in the current vaccine (477).

There is little information about the persistence of antibody after this single dose priming regime and subsequent memory responses. Infants immunized at 2, 4, and 6 months of age generate IgG antibody responses to the Pnc7 vaccine (187), but the serum antibody wanes rapidly, with some serotype specific antibody levels falling below the protective threshold within a matter of months (157, 477). Similarly, antibody wanes rapidly after immunisation with other glycoconjugate vaccines in early infancy such as *Haemophilus influenzae* type b (Hib) (478) and serogroup C *Neisseria meningitidis* glycoconjugate vaccine (MenC) (479), and there is a corresponding loss of vaccine effectiveness (480, 481). This failure of persistence of IgG to capsular polysaccharides after immunisation in infancy may be overcome by the subsequent administration of a booster dose of a conjugate vaccine at 12-15 months of age, a marked rise in IgG antibody levels demonstrating that immunological memory had been induced by priming (179, 482, 483). Therefore, despite the below adequate levels of circulating antibody, most infants have developed immune memory in response to primary immunisations with glycoconjugate vaccines.

By contrast, in young adults a single dose of Pnc7 vaccine is sufficient to induce protective levels of IgG to all seven serotypes included in the heptavalent vaccine, although the levels also wane somewhat (101, 159, 181, 410) and no further increase in response is demonstrated following re-immunisation (267) possibly because polysaccharide antigens (conjugated as well as purified), appear to stimulate predominantly marginal zone B cell responses in this age group (123, 252, 267, 411).

The role of the different B cell subsets involved in response to the vaccine components becomes more of an issue when comparing different age groups. B1 B cells are present early in foetal development and are known to mediate innate immune responses rather than participate in vaccine specific responses. It is the roles of the FO and MZB cells that are more likely to differ between age groups. MZB cells accumulate with age and require a mature splenic marginal zone to function and can undergo rapid isotype switching to IgG^+ during the first week after immunisation (307). It is the appearance of mature MZB that coincides with the ability of individuals to respond to polysaccharide antigens. MZB have little or no role in infant immune responses before 12 months of age since there are few and they are immature. This feature of the infant immune system may be a contributing factor in the lack of long term maintenance of serum IgG levels in infants (317). Responses to polysaccharides in this age group are mediated by FO B cells that require T cell help to respond to primary exposure to polysaccharide antigens. This is the reason for the lack of immune memory in infants immunised with plain polysaccharide. In young adults the role of FO B cells in the response to polysaccharide antigens is mitigated by the preferential localisation of complement opsonised polysaccharide to the splenic marginal zone. Fully mature marginal zone B cells bind the opsonised polysaccharide via surface bound complement receptor-BCR complex. This co-ligation of receptors leads to enhanced signalling to the B cell, negating the need for T cell co-stimulation to instigate B cell proliferation and differentiation. Infant MZB cells lack the expression of the complement receptor CD21, a surface

receptor for C3d component that is known to mediate binding of opsonised pneumococcal polysaccharides by B cells and fully mature MZB cells have not been detected until two years of age. While increasingly mature MZB cells allow immune responses to polysaccharides to develop, conjugation of the polysaccharide would not alter this response.

Immunisation against pneumcoccal disease in the elderly is recommended above the age of 65 years in the UK and involves a single dose of 23 valent purified polysaccharide vaccine (23PsV). While the conjugate vaccine is efficacious in infants, there is little evidence that it would be of benfit to replace the existing polysaccharide vaccine with the conjugate. At this extreme of the age range, MZB cells are present, but after the age of forty years old the frequency steadily declines (figure 1.4 of introduction from Y. Shi *et al* figure 6 (250) and S. Weller *et al* (252, 253)).

The efficacy of 23PsV is not good though and several studies have shown that in the long term the benfits of immunisation are poor (90, 484-486). However, in young healthy adults the efficacy of 23PsV is better, inducing protection against invasive disease and pneumonia (90, 487). The benefit of pneumococcal conjugate vaccines over 23PsV in the elderly is unclear and had been little studied since 23PsV was thought to be efficacious (488). Also, Pnc7 only covers around 56%-66% of disease causing serotypes in the >65's (64, 488). Responses in the infants and young adults are boosted by prior exposure to pneumococci through nasopharyngeal carriage, but carriage decreased with increasing age (489), so the potential augmentation of the response to the conjugate vaccine may be reduced in the elderly. Also it has been shown that natural anti-pneumococcal immunity in the elderly is mediated through antibody against the cell wall polysaccharide (CwPS), and Sankilampi *et al* (487) showed that 99% of >65's had anti-CwPS IgG antibody in their serum, while capsule specific antibody decreased with age. The benfits of a single dose of conjugate are un-certain and comparison with a single dose of 23PsV revealed similar responses in serum IgG (90), but multiple doses did improve the response. Powers *et al*

(172) had previously shown that a single dose of a pentavalent-CRM conjugate had no benefit over the polysaccharide and that boosting a single dose of conjugate with a dose of 23PsV had no effect.

One reason for the poor efficacy of the new conjugates in the elderly may lie in impaired T cell immunity. Studies in mice have revealed that TD-responses are impaired in the elderly compared to young adults. The IgG response to the TI-antigen (purified polysaccharide from serotype 14), was equivalent in the young and elderly adults while the IgG response to serotype-14-PspA conjugate was impaired in the elderly (409). Before this study, Hwang *et al* (490), showed that blockade of CD40L by a monoclonal antibody reduced the murine IgG response to both the capsular polysaccharide and PspA components of the conjugate but the response to the CwPS remained intact. Both of these studies suggested that elderly T cell responses where impaired, affecting the B cell response to the polysaccharide conjugate but not the innate immune response to CwPS, and thus possibly accounting for high levels of antibody against CwPS in this age group while there remained concurrent low levels of capsule specific antibody (491). T cell responses in the elderly may be impaired through increased clonality of the memory population through chronic latent infections by viruses such as cytomegalovirus (CMV) and Epstein Barr virus (EBV) (492). A study of responses to influenza vaccine in the elderly revealed that CD4+T cell responses were similar in the elderly and young adults, but that the elderly failed to maintain a population of effector memory T cells (T_{EM}) (140). The $CD4^+$ T_{EM} population is replenished from the central memory population (493), so increasing clonality of the central memory population with age would impair antigen specific T cell responses in the elderly. Lack of T cell responses to immunisation would account for the poor quality of antibody produced in this age group with conjugates as a result of poor affinity (105) and germinal centre formation, decreased somatic mutation rates and defective generation of long lived plasma cells (409).

The age groups chosen in this study should reflect these differences in the frequencies of B cell populations involved in the immune response to pneumococcal polysaccharides. There may also

be differences in the *in vitro* B cell response between the age groups and between polysaccharide verses conjugate vaccines.

6.3 Aims

The aim of this study was to investigate the capsular polysaccharide specific B-cell response following immunisation of different age groups with Pnc7 conjugate vaccine. The groups chosen were 12-month old toddlers, young adults and elderly adults.

The second aim of this study was to determine the differences in the serum antibody response to the polysaccharide component of the Pnc7 immunisation in adults and toddlers. The final aim was to see whether the responding B cell subsets differed between the young and elderly adults and whether administration of a conjugate vaccine had any benefit over the polysaccharide vaccine in inducing B cell subset responses in the elderly.

6.4 Subjects and clinical procedures

Details of the subjects, vaccine and blood draw schedules for each of the groups are summarised in table 6.1. The number of samples obtained for each part of the three age group studies is shown in table 6.2.

6.4.1 Toddler Study

Forty healthy toddlers, aged 12-months (see table 6.1), received 2 doses of heptavalent pneumococcal-CRM_{197} conjugate vaccine at a 2 month interval. A 5ml venous blood sample was collected (1ml into a clotted tube for serum and 4ml into a heparin tube for isolation of PBMCs), prior to vaccination, on days 6 or 7 following the primary dose, on day 56 (prior to the second dose), and finally 6 or 7 days following the second immunisation.

6.4.2 Young adult study

Twenty healthy adult volunteers with a mean age of 32 years, (see table 6.1), with no previous history of pneumococcal vaccination, received a primary dose of the heptavalent pneumococcal-CRM_{197} conjugate vaccine (Pnc7, Wyeth Vaccines, Pearl River, MA) by intra-muscular injection in the left deltoid. The 0.5ml dose of the vaccine contained a concentration of polysaccharides of 2.0μg/ml for each of serotypes-4, 9V, 4, 18C, 19F, 23F and 4μg/ml of 6B. Each polysaccharide is conjugated to CRM_{197} (mutant diphtheria toxoid) and adsorbed on aluminium phosphate. A 20ml venous blood sample was collected (2ml into a clotted tube for serum and 18ml into a heparin tube for isolation of peripheral blood mononuclear cells (PBMCs), prior to vaccination and again on days 6, 7, 15 and 28 following immunisation.

Informed consent for both the young adult and toddler studies was obtained from the volunteers and the protocol was approved by the Oxfordshire Research Ethics Committee (OxREC number C02.005).

6.4.3 Elderly adult study

A group of 27 elderly adults with a mean age of 59 years (see table 6.1), were recruited as a subgroup of part of a larger study, and were randomised to receive a single dose of Pnc7 conjugate vaccine (n=18) or 23PnV polysaccharide vaccine (n=9) with a 20ml heparinised blood sample obtained prior to and 28 days after immunisation. The samples were blinded with coded laboratory numbers so that the vaccine group was unknown until statistical analysis.

Informed consent was obtained from the volunteers and the study was approved by the Oxfordshire Research Ethics Committee (OxREC number OVG2006/01).

Group	recruitment age mean (range)	n	sex (F)	Vaccine (no. of doses)	vaccinations (V) and blood samples (B) day 0	day 6	day 7	day 15	day 28	day 56	day 62/63
young adults	32 (23-49)	20	12	Pnc7 (1 dose)	B+V	B	B	B	B		
elderly	59 (47-66)	18	12	Pnc7 (1 dose)	B+V				B		
adults	56 (50-65)	9	6	23PsV (1 dose)	B+V				B		
toddlers	12-months	40	25	Pnc7 (2 doses) *2 month interval*	B+V	B(d6/7)				B+V	B

Table 6.1 Details of the subjects recruited, vaccines and time points of vaccinations and blood samples.
The table shows the age of each study group, the number (n) recruited and proportion that were female (F). The vaccines (V) given were either a heptavalent pneumococcal CRM197 conjugate vaccine (Pnc7) or 23-valent pneumococcal polysaccharide vaccine (23PsV). The young adults received a single dose of Pnc7 and blood (B) was obtained prior to and on days 6, 7, 15 and 28 after immunisation. The elderly adults were randomised to receive a single dose of either Pnc7 or 23PsV with blood samples obtained prior to and 28 days following vaccination. The toddlers received two doses of Pnc7, the first at 12 months of age and the second 2 months later, at 14 months of age. Blood was obtained prior to and on days 6 or 7 after the first dose, on day 56 (prior to the second dose) and one week after the second dose (d62 or 63 of the study). The greyed out boxes are time points not included in each study.

Group	vaccine given	study day	ELISA IgG/IgA/IgM	ELISpot IgG-AFC ex-vivo	ELISpot IgG-AFC Memory	CFSE
young adults	Pnc7	0	20	7	10	10
		6	20	10	10	10
		7	20	10	10	10
		15	20	7	10	10
		28	20	n/a	10	10
elderly adults	Pnc7	0	n/a	n/a	18	18
		28	n/a	n/a	18	18
	23PsV	0	n/a	n/a	9	9
		28	n/a	n/a	9	9
toddlers	Pnc7	0	40	19	36	n/a
		6/7	40	31	24	n/a
		56	40	13	33	n/a
		62/63	40	31	23	n/a

Table 6.2 The number of samples obtained and tested for each parameter at each time point.
In the young adult study serum was collected for quantification of serum IgG, IgA and IgM concentrations to serotypes 4, 14 and 23F. PBMCs were collected and the frequency of IgG-AFC were quantified in the *ex vivo* and memory B cell ELISpot assays. The proliferation o f B cell subsets in the SAC+IL-2 versus SAC+CpG+PWM culture systems was also analysed by CFSE dye dilution. In the elderly adult study the frequency of memory B cell derived IgG-AFC was quantified along with B cell subset proliferation by CFSE dye dilution. In the toddler study the serum IgG, IgA and IgM concentrations to serotypes 4, 14 and 23F were quantified and PBMCs were collected for the quantification of both memory B cell derived and *ex vivo* IgG-AFC.

6.5 Materials and methods

Serum IgG, IgA and IgM ELISAs, *ex vivo* and memory ELISpots were performed in the study samples from the young adults and toddlers and memory ELISpots alone were undertaken in the study of the elderly adults. B cell proliferation studies were carried out using CFSE analysis on PBMCs from both the elderly and young adults. The serum antibody levels for the elderly adult group were sent to the Health Protection Agency in Manchester for analysis by multiplex serotype analysis by Luminex^{TM}. Data for these serum samples was not available for this thesis and the method was different from the ELISA used to analyse the samples from the young adult and toddler groups.

The antibodies used in the ELISA and ELISpot assays are listed in chapter 2, section 2.1. The buffers used are listed in chapter 2, section 2.2. PBMCs were separated as described in chapter 2, section 2.4. Following the studies discussed in chapter 5 the stimulation of the PBMCs for the inter age comparison of IgG-AFC frequencies was carried out using SAC+CpG+PWM as described in chapter 2, section 2.6 and the cells harvested as in section 2.7.

The ELISPot assays for all age groups was undertaken as described in chapter 2, section 2.5 and the serum antibodies were measured as described in chapter 2, section 2.10.

For the B cell proliferation studies in the young and elderly adults the PBMCs were labelled with CFSE as described in chapter 2, section 2.14. *In vitro* stimulation of the labelled PBMCs was carried out using SAC+IL-2 and SAC+CpG+PWM as described in chapter 2, section 2.6 and phenotyping of the cell surface markers as in section 2.8.

6.6 Statistics

Comparisons of cell frequencies, obtained by ELISpot, between individuals of the same age group at each time point were carried out using the Wilcoxon Signed Rank test.

Comparisons of cell frequencies between the age groups were analysed using the Mann Whitney Test in SPSS v12 and Graph pad Prism V4.0. Log transformation of ELISA data and calculation of geometric mean concentrations (GMC) was carried out using Microsoft Excel statistical functions. The comparisons within and between age groups were carried out using the paired and unpaired TTEST function respectively.

6.7 Results

6.7.1 The *ex vivo* IgG-AFC response in 12 month old toddlers given a primary (day 0) and secondary dose (day 56) of Pnc7.

For all of the antigens tested except diphtheria toxoid there was at least one individual who had some *ex vivo* AFC on day 0, prior to immunisation (Figure 6.1a-e). However, at days 6/7 following immunisation there was a significant rise in frequency of AFC in response to diphtheria toxoid (dip) and serotypes-4, 14 and 23F ($p=0.001$, $p=0.002$, $p=0.007$, $p=0.013$ respectively), fig.6.1b-e. The overall AFC response 1 week after immunisation was high with between 74% (serotype 23F) and 97% (dip) of toddlers demonstrating an increased frequency of IgG-AFC specific for the vaccine antigens, and only 6.5% for tetanus toxoid. The strongest responses following the primary dose of Pnc7 were to diphtheria toxoid and serotype-4 (median spot number of 40 and 30 spots/10^6 PBMCs respectively, fig.1b-c), with >90% responding. Serotype-14 and 23F induced a lower response with a median number of 10 spots/10^6 PBMC (fig.6.1d-e). Following re-immunisation of the toddlers with a second dose of Pnc7 on day 56 after the first dose, there was no significant difference in the overall magnitude of the secondary response over the primary response 1 week after immunisation (fig 6.1b-e). The number of responders to the polysaccharide component of the vaccine dropped by 24%, 14% and 7% for serotypes-4, 14 and 23F respectively, following the second dose of vaccine. The *ex vivo* response to diphtheria toxoid

was also lower than after the first dose, (p=0.002), with the number of responders decreasing from 93.5% to 80%.

6.7.2 The *in vitro* induced IgG-AFC response in 12 month old toddlers given a primary (day 0) and secondary dose (day 56) of Pnc7.

In vitro stimulation of B cells isolated from the peripheral blood allowed the detection of IgG-AFC derived from memory B cells. The memory B cell derived AFC frequency to tetanus toxoid (a non-vaccine related antigen), remained unaltered throughout the study time course (fig.6.1f). Also 86% of the toddlers already had diphtheria specific memory cells while only 2.5-5.6% had any polysaccharide specific memory prior to immunisation. Seven days after the primary Pnc7 immunisation of toddlers, there was a significant increase in the frequency of *in vitro* inducible, antigen specific AFC, derived from peripheral blood memory B-cells (fig. 6.1 g-j). The median number of spots at days 6/7 was 70, 10, 5 and 10 per 10^6 cultured PBMCs for diphtheria, serotype-4, 14 and 23F respectively. The percentage of toddlers with detectable serotype-14 and 23F specific memory B-cells increased to almost 70%, and >90% for serotype-4 and diphtheria toxoid. By day 56 the frequency remained significantly elevated or even increased further above baseline levels (63-91% showing polysaccharide memory) (Fig 6.1g-j). On day 56, administration of the second Pnc7 dose, generated a further increase in inducible, polysaccharide specific AFC one week following immunisation (d62/63), with 86.9 to 95.6% being responders. However, the response to diphtheria toxoid appeared to reach a plateau following the primary dose and was not improved by re-immunisation (fig 6.1g). The resulting memory response 1 week post secondary immunisation appeared to be most prominent to serotype-4, followed by 23F and 14 (also represented by the percentage of individuals responding to immunisation).

6.7.3 The effect of age on the *ex vivo* IgG-AFC response to immunisation with Pnc7 vaccine

The frequency of spontaneously secreting, antigen specific AFC was significantly greater in adults than in the 12-month old infants as measured seven days after administration of a primary dose of Pnc7-CRM conjugate vaccine (fig 6.2a, p=<0.0001).

Administration of a second dose of conjugate vaccine did not alter this difference in frequency which remained highly significant for all antigens (diphtheria, serotype-4, 14 and 23F, fig 6.2b).

6.7.4 The effect of age on the memory B cell derived IgG-AFC response to immunisation with Pnc7 vaccine.

Total IgG secreting cells (fig. 6.3a), were enumerated as a control for the *in vitro* differentiation of AFC from peripheral blood memory B-cells. At all time points studied the median frequency of total IgG-secreting cells was significantly lower in infants than in young adults, although the median frequency was constant within each of these two groups (fig. 6.3a). The infants were re-immunized 56 days after the first dose of Pnc7 (black arrow,fig 6.3a). The frequency of total IgG secreting cells was unaltered one week after the second dose (d63 in fig 6.3a), compared to that at day 56 or day 0.

There remained a significant difference, between the age groups, in antigen specific memory B cell frequency specific for serotype-4, 14 and 23F (p=0.002, <0.001, 0.009 respectively, fig.6.3b), on day 7 after the primary dose of Pnc7. Adult responses were higher with the exception of diphtheria toxoid, in response to which the toddlers and young adults mounted equivalent responses (p=>0.05).

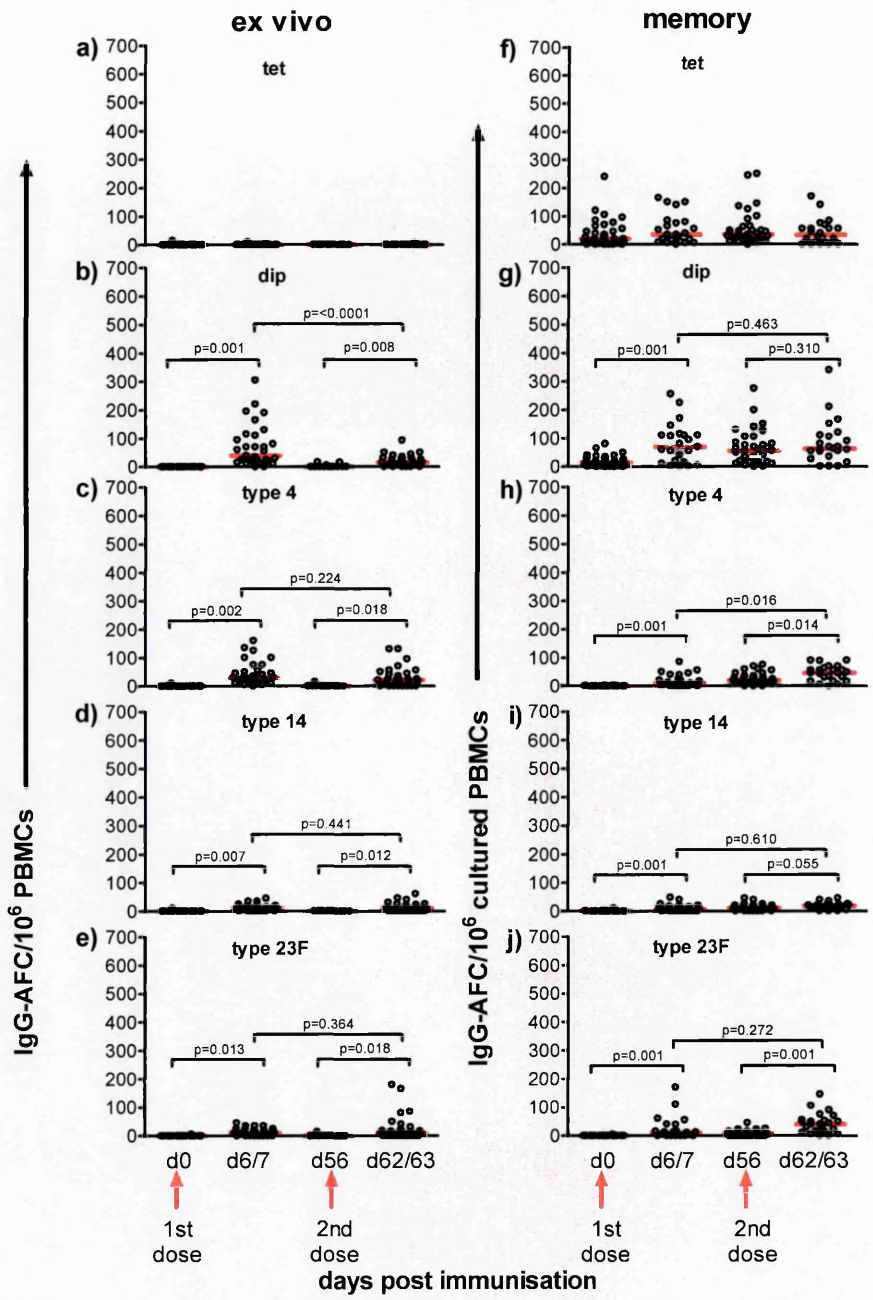


Figure 6.1 The frequency of spontaneously secreting IgG-AFC and memory B-cell derived IgG-AFC. PBMCs were isolated from the peripheral blood of 12 month old toddlers prior to a first dose (d0) and second dose (d56) of Pnc7 (shown by the red arrows). PBMCs were also obtained one week after each dose on days 6/7 or 62/63. At each time point the frequency of plasma cells were quantified in the *ex vivo* ELISpot (a-e). PBMCs from the same samples were also stimulated for 5 days with SAC+CpG+PWM to detect memory B cell derived IgG-AFC (f-j) after which they were harvested and allowed to secrete IgG onto well of an ELISPot plate. The ELISpot wells were coated with tet, dip, and serotypes 4, 14 and 23F polysaccharides. Data are expressed per 10^6 PBMCs or cultured PBMCs and the red line shows the median frequency.

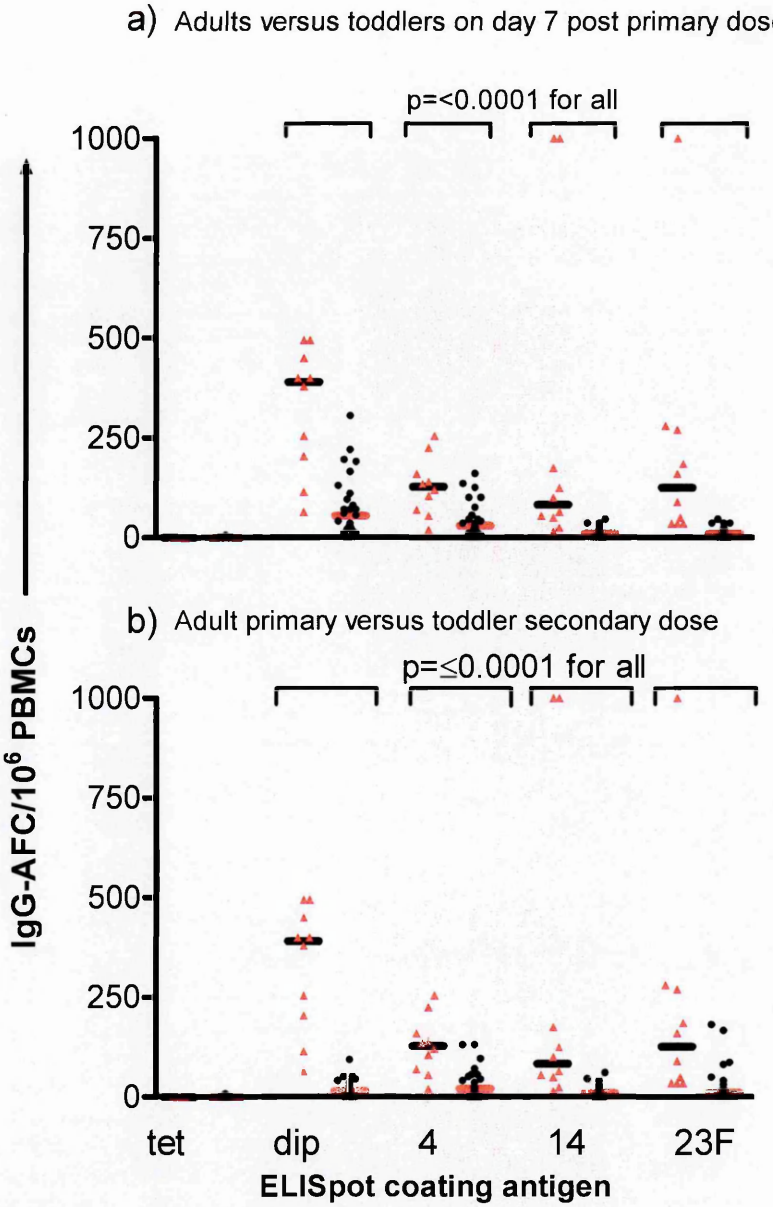


Figure 6.2 The frequency of spontaneously secreting IgG-AFC, isolated from the peripheral blood of adults (red triangles) and 12 month old toddlers (black circles) were compared.
PBMCs were isolated seven days after a primary or secondary dose of Pnc7 and seeded into antigen coated ELISPot wells for the detection of IgG-AFC specific for tet, dip, serotype 4, 14 and 23F. (a) Compares the IgG-AFC frequency after a primary dose in both adults and toddlers. (b) Compares the primary dose response in adults with that of toddlers seven days after their second dose of Pnc7. The data are expressed as the IgG-AFC/10^6 PBMCs for each individual and the black bar shows the median frequency for each group. The inter age group comparison was analysed using the Mann Whitney U test for un-paired, non parametric data.

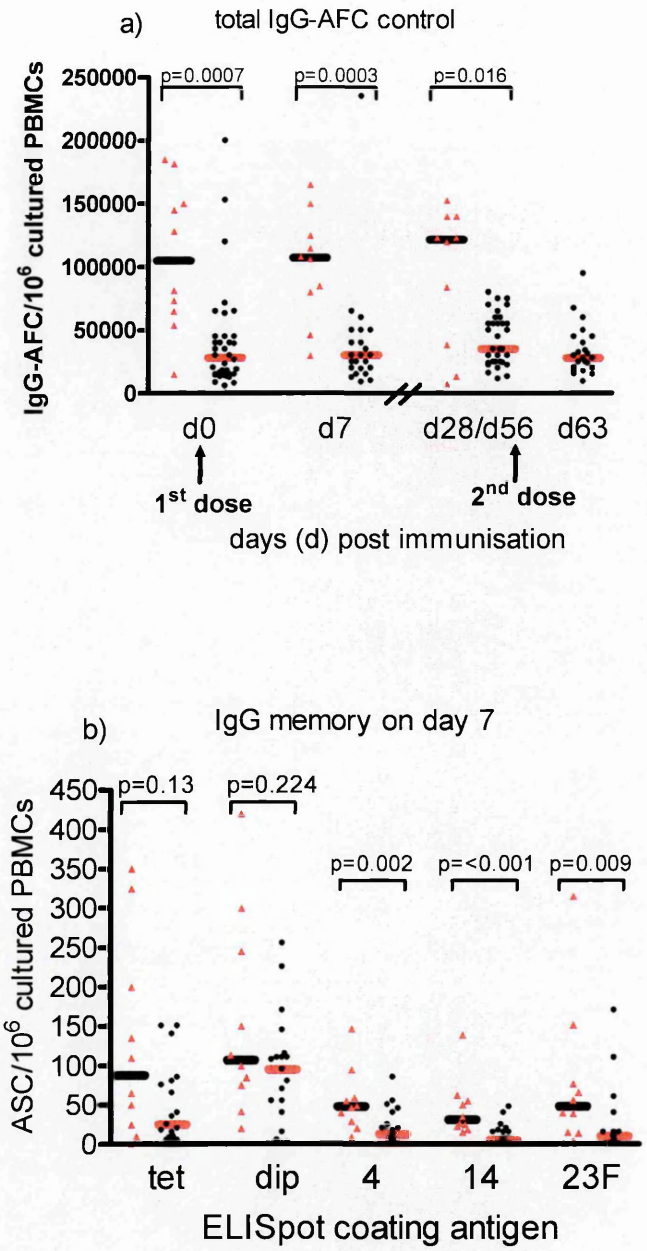


Figure 6.3 The frequency of memory B cell derived IgG-AFC isolated from the peripheral blood of adults (red triangles, n=10) and 12 month old toddlers (black circles, n=29).
PBMCs were isolated prior to (d0) and seven days after (d7) administration of a single dose of Pnc7 for adults and toddlers. Two months later (d56 post primary) the toddlers received a second dose of Pnc7. PBMCs were isolated before (d56) and seven days after the second dose (d63 post primary dose). The PBMCs at all time points were stimulated for 5 days in the presence of SAC+CpG+PWM, and then harvested and seeded on to antigen coated ELISpot wells were they were left to secrete IgG overnight. a) ELISpot wells coated with anti-human immunoglobulin were used as the control for IgG-AFC formation. b) Antigen specific IgG-AFC were detected in antigen coated wells. The data are expressed per 10^6 cultured PBMCs and the bars show the median frequency for each antigen.

6.7.5 The time elapsed since immunisation and its effect on detection of memory B cell derived, antigen specific IgG-AFC in young adults and toddlers.

There was no significant difference in IgG-AFC frequency for serotype 4 and diphtheria or tetanus toxoid, between adults on day 28 and toddlers on day 56 (fig 6.4a). However, IgG-AFC specific for serotype-14 and 23F remained significantly lower in frequency in the toddlers than in adults at these time points.

Toddlers received a second dose of Pnc7 two months (d56, 14 months of age) after the first dose. One week following the second dose of conjugate (day 63 of enrolement), blood was drawn and the memory B cell derived AFC frequency quantified (fig.6.4b). Toddlers attained levels of memory B cells equivalent to those achieved by adults one week after a primary dose. This was the case for all antigens except serotype-14 (fig 6.4b), to which the response remained significantly lower in the toddlers ($p=0.004$). When the toddler response one week after the secondary response was compared with the young adult response on day 28 after the primary dose, no significant difference in memory B cell derived IgG-AFC frequency was found in response to any of the antigens tested (fig 6.4c).

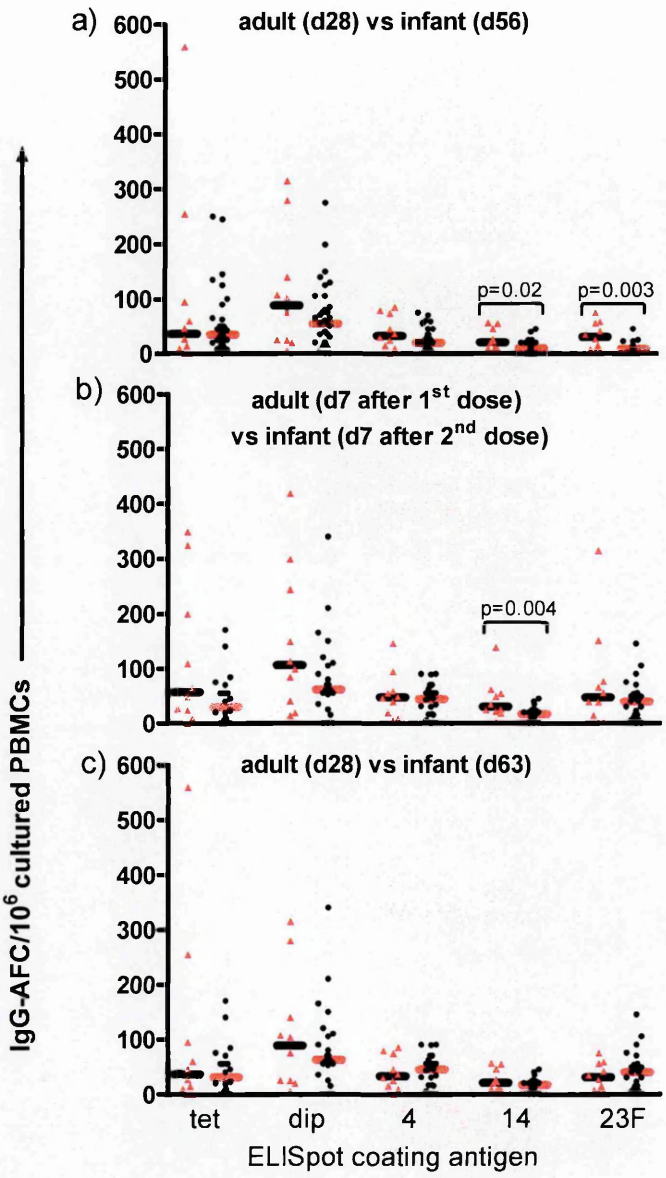


Figure 6.4 A comparison of memory B cell derived IgG-AFC from adults (red triangles) and 12-month old toddler (black circles) peripheral blood.
PBMCs were isolated and cultured for 5 days in the presence of SAC+CpG+PWM before enumeration of IgG-AFC by ELISpot specific for tet, dip, serotype 4, 14 and 23F polysaccharides. The frequency of memory B cell derived IgG-AFC were compared between adults (d28) and toddlers (d56) to see the longer term effect of a single dose of Pnc7 on frequency (a). The frequency of IgG-AFC 7 days after a single Pnc7 dose in adults was compared with that from toddlers seven days after a second dose (b). The adult memory B cell derived IgG-AFC frequency at d28 post primary was compared to that seen in toddlers seven days after the second dose of Pnc7 (d63) (c). Data are expressed as the IgG-AFC/10^6 cultured PBMCs and the bars show the median frequencies in adults and infants.

6.7.6 The effect of increasing age on response of peripheral blood memory B cells to Pnc7 immunisation of young and elderly adults.

Prior to immunisation there was a significant difference in the frequency of the total IgG-AFC between the age groups (fig.6.5a). The pre-existing memory B cell derived IgG-AFC frequency in the elderly adults was significantly lower with a median spot number of 25,938/10^6 cultured PBMCs, (0.026%), versus 105,000/10^6 cultured PBMCs, (0.105%), in the younger age group. Following immunisation the difference in frequency remained but was not significant (p=0.303) with a median spot count of 54,165/10^6 cultured PBMCs, (0.054%), in the elderly and 121,675/10^6 cultured PBMCs, (0.12%), in the younger age group.

Twenty-eight days after immunisation with a single dose of the pneumococcal conjugate vaccine, both young and elderly adults achieved equivalent frequencies of antigen specific IgG-AFC (fig.6.5b-c). The median frequencies of spots/10^6 cultured PBMCs in the elderly vs young adults were as follows: Tetanus (47 vs 53, p=0.848) and diphtheria (21 vs 106, p=180); Serotype-4 (20 vs 40.8, p=0.108), serotype-14 (21.5 vs 25.3, p=0.980), serotype-23F (20 vs 33, p=0.533).

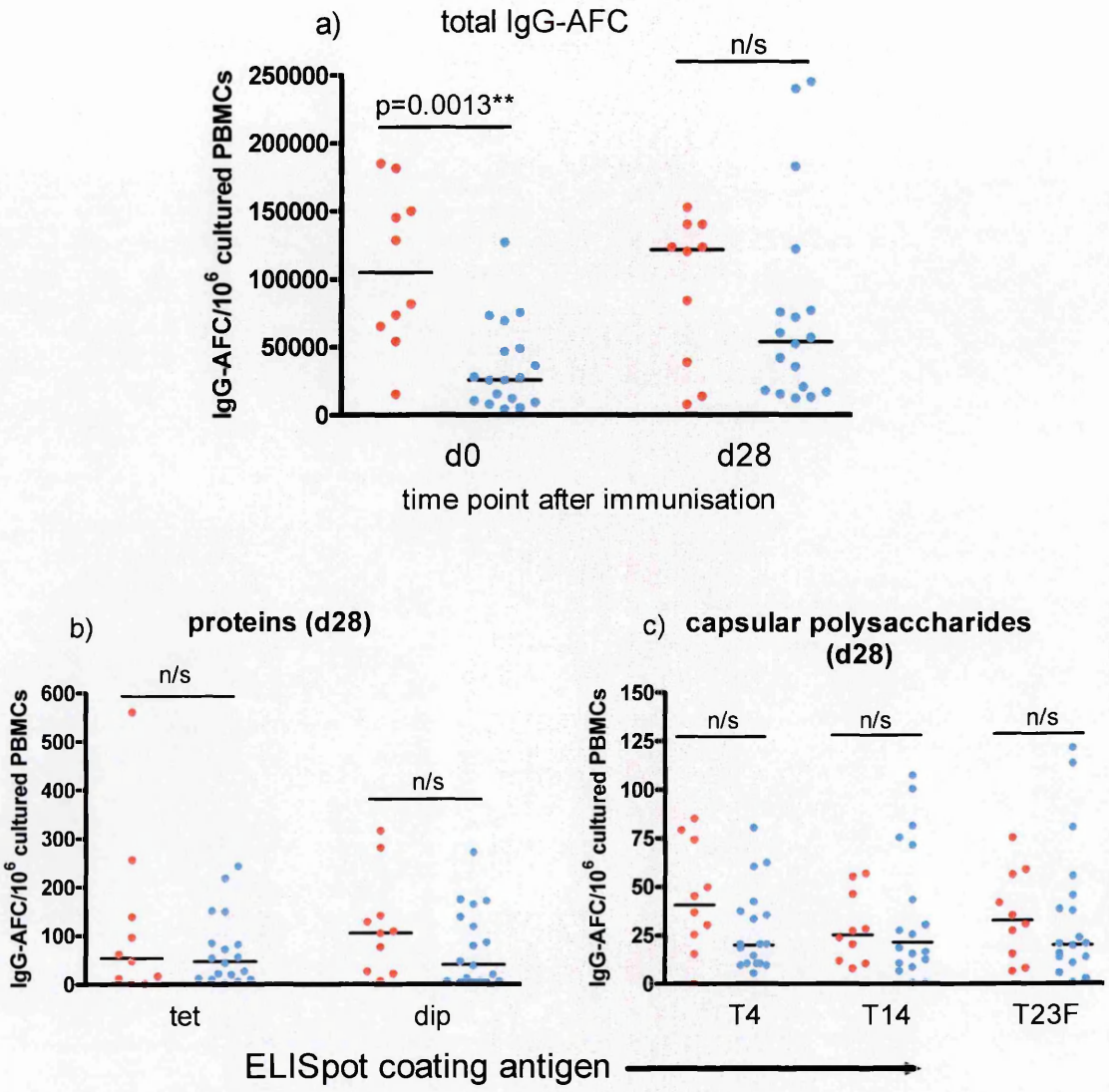


Figure 6.5 A comparison of the frequency of memory B cell derived IgG-AFC isolated from the peripheral of young adults (red, n=10) and elderly adults (blue, n=18) prior to (d0) and 28 days (d28) after immunisation with a single dose of Pnc7 vaccine.

The PBMCs were cultured for 5 days with SAC+CpG+PWM. After 5 days the cells were harvested and seeded onto antigen coated ELISpot plates specific for total-IgG (fig.6.5a), tetanus (tet) and diphtheria (dip) toxoid (fig.6.5b), and pneumococcal capsular polysaccharides from serotypes 4, 14 and 23F (fig.6.5c). The data represent the number of IgG-AFC/10^6 cultured PBMCs with the median frequency represented by the black bar.

6.7.7 The effect of age on the baseline frequency of memory B cell derived IgG-AFC in toddlers, young and elderly adults.

There was a slight difference in the frequency of tetanus specific memory IgG-AFC between the elderly adults and infants (Fig.6.6a, p=0.022) but no difference in diphtheria toxoid specific IgG-AFC frequency (21 vs. 5 vs 14 spots/10^6 cultured PBMCs respectively), as is seen in fig. 6.6b).

In the case of pneumococcal serotype specific baseline memory cell frequency (fig.6.6c-d), young adults demonstrated the highest frequencies of polysaccharide specific memory IgG-AFC. The median IgG-AFC frequency was significantly higher in the young adult group than in the elderly or toddler group (fig.6.6 c-d). The elderly group demonstrated significantly higher baseline B cell memory to the polysaccharide antigens than that found in the toddlers (fig 6.6.c-d).

For the capsular polysaccharides of serotypes-4, 14 and 23F the median spot counts for the young adults vs elderly adults vs infants were (26 vs. 2 vs 0), (23 vs. 4 vs. 0) and (27 vs. 2 vs. 0 IgG-AFC/10^6 cultured PBMCs respectively).

6.7.8 The toddler anti-polysaccharide antibody responses following primary and secondary doses of Pnc7.

A single dose of Pnc7 in 12-month old toddlers led to a significant increase in IgG, IgA and IgM anti-polysaccharide antibodies to each of the three serotypes tested at one week after immunisation (table 6.3).

By day 56 (2 months post immunisation), IgG levels had increased significantly above the d6/7 levels for serotypes-4 and 14 (GMC 2.16μg/ml and 2.78μg/ml respectively). A single dose of Pnc7 did not appear to generate such a good response to 23F during the same time period, although the IgG level of 0.33μg/ml was above one postulated threshold for protection (>0.2μg/ml). IgA and IgM levels over the same 2 month period declined from day 6/7 levels, though remained above baseline.

A second dose of Pnc7 induced a significant increase in IgG, IgA and IgM to all three serotypes, with serotype-14 generating the greatest IgG response and 23F the least. The serotype 14 and 23F response consisted almost equally of IgG and IgM.

The primary dose induced more polysaccharide specific IgM than IgG to all three serotypes. By day 56 this was still the case for serotype-14 and 23F, while the serotype-4 response appeared to have switched to a more IgG mediated response. Following the second dose of Pnc7 IgG appeared to be the main mediator of the response to serotypes 4, 14 and 23F.

6.7.9 Serum anti-pneumococcal antibody in adults and toddlers.

Prior to immunisation the adults had significantly more serum IgG antibody to serotypes-4, 14 and 23F than that detected in the 12-month old toddlers (fig 6.7a). This was also the case for serum IgA (fig 6.7b). However, serum IgM was only significantly different for serotypes-4 and 14 (fig.6.7c). Similar levels of IgM against serotype 23F were present in both age groups ($p=0.590$, fig. 6.7c). The percentage contributions of IgG, IgA and IgM were noticeably different between adults and toddlers prior to immunisation (d0, fig.6.9). IgM dominated the serum antibody response to all three serotypes in toddlers, while IgG contributed 43%-66% of the total antibody response in adults to each serotype.

At seven days following the primary dose of Pnc7 the GMC of serotype 4 specific IgG was the same in both age groups ($p=0.544$, fig.6.8a), while there was still significantly higher concentrations of IgG to serotype-14 and 23F in adults ($p=<0.0001$ in both cases fig.6.8a). There were no significant differences in the levels of IgA generated by the first dose of Pnc7 in the two age groups to any of the serotypes (fig.6.8b, $p=0.149$, 0.124 and 0.056 respectively for serotypes 4, 14 and 23F). This was also true for IgM induced by serotype-4 (fig.6.8c, $p=0.199$). There was

a trend for higher IgM to serotype-14 in toddlers and a significantly higher level of 23F IgM compared to adults (p=0.088 and p=0.026 respectively, see figure 6.8c).

The percentage contribution of each isotype to the overall response 1 week after a single dose was similar for serotype-4 in both adults and infants (fig 6.9). IgM still dominated the infant antibody response to serotypes-14 and 23F.

The adults B cell and antibody response at d28 was compared with the toddler response on day 56 after a primary dose of Pnc7 to look at the development of the IgG response. Ethical constraints did not allow for an extra toddler sample at day 28. By day 56 in toddlers verses day 28 in adults the contribution of IgG in the response to all three serotypes had increased further in toddlers (fig 6.9). The response to serotype 4 was now the same as that in adults while the responses to serotypes 14 and 23F were still mediated more by IgM than in adults (fig 6.9).

Following the second dose of Pnc7 in toddlers there were no significant differences in the GMC of serum IgG when compared with adults one week after their first dose (fig.6.8d). This was also true for serum IgA levels to each of the capsular polysaccharides (fig.6.8e).

The IgM response to the second dose in infants induced significantly higher levels of IgM for serotypes 14 and 23F in the infants (fig 6.8f), while for serotype 4 the levels were equivalent. Comparing the contributions of IgG, IgM and IgA to the serum antibody response in toddlers at 1 week after a secondary dose of conjugate with adults 1 week after a primary dose revealed the following. The response to serotype 4 in infants was now 79% IgG vs 37% in adults (fig.6.9). The percentages of the isotypes induced by serotype 14 were now the same in adults and infants. Serotype 23F was still inducing a split IgG/IgM response in infants (49%/45% respectively), while in adults after one dose the response of IgG/IgM was 68%/24% respectively (fig 6.9).

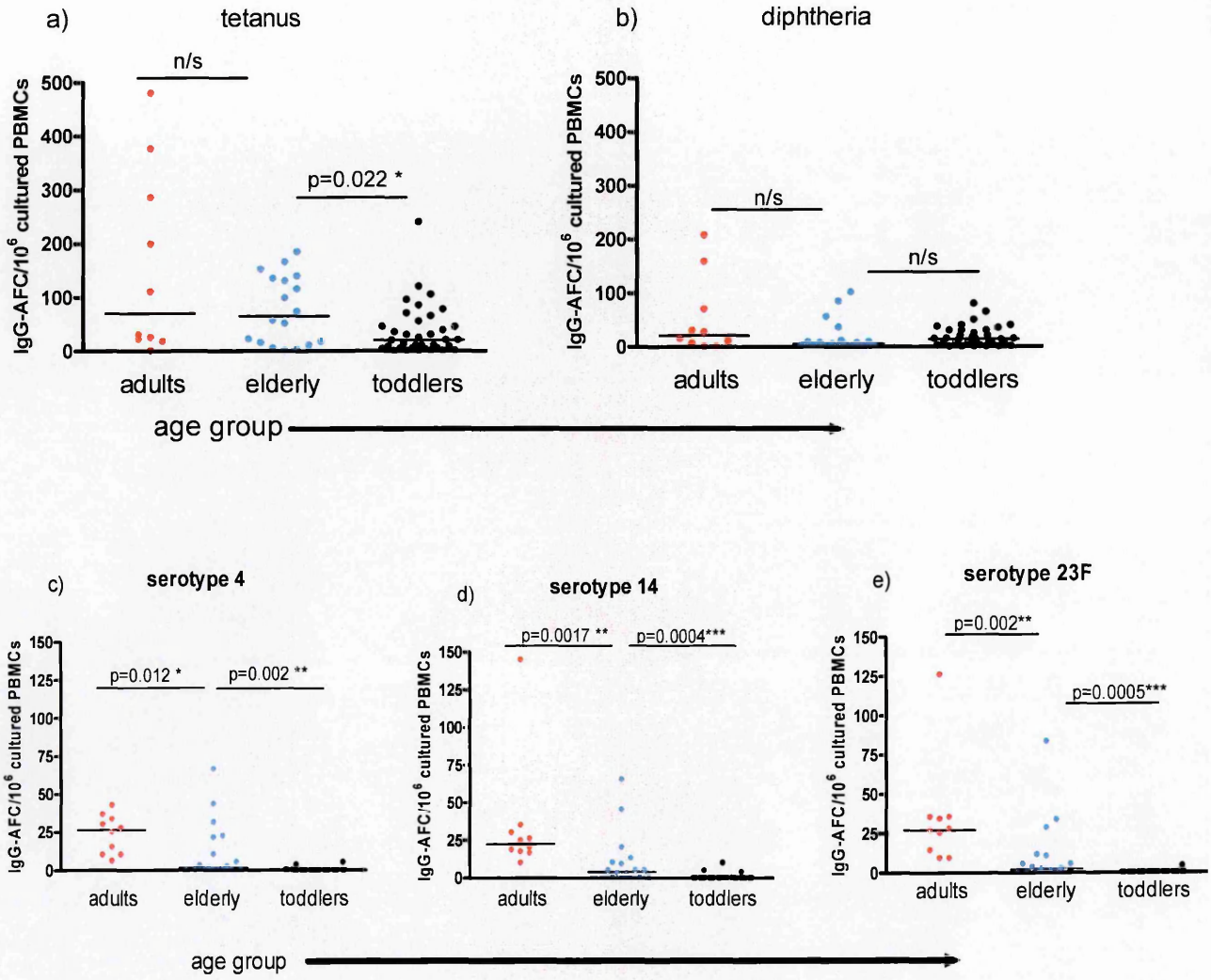


Figure 6.6 The effect of age on the baseline (d0) frequency of protein and polysaccharide specific memory B cell derived IgG-AFC.
PBMCs were isolated from the peripheral blood of young adults (red, n=10), elderly adults (blue, n=18) and toddlers (black, n=36). The PBMCs were then cultured for 5 days in the presence of SAC+CpG+PWM. Following stimulation the cells were harvested and the frequency of antigen specific IgG-AFC determined by ELISPOT specific for tetanus (a), diphtheria (b) serotype 4 (c), 14 (d), and 23F (e). The data are expressed as the number of IgG-AFC/10^6 cultured PBMCs and the bar shows the median value for each of the studies.

		time point after primary immunisation (days)			
		first dose		second dose	
serotype		0	6/7	56	62/63
4	IgG	**0.02** (0.01-0.04)	***0.77** (0.52-1.16)	***2.16** (1.65-2.83)	***6.23** (4.42-8.78)
	IgA	**0.02** (0.01-0.02)	***0.49** (0.34-0.71)	***0.20** (0.14-0.71)	***0.70** (0.51-0.97)
	IgM	**0.14** (0.11-0.17)	***1.12** (0.85-1.47)	***0.54** (0.42-0.68)	***0.98** (0.75-1.29)
14	IgG	**0.05** (0.02-0.10)	***0.66** (0.38-1.14)	***2.78** (1.72-4.50)	***12.11** (9.44-15.55)
	IgA	**0.02** (0.01-0.03)	***0.28** (0.20-0.40)	***0.15** (0.11-0.21)	***0.81** (0.62-1.07)
	IgM	**1.22** (0.98-1.52)	***5.90** (4.65-7.50)	***4.10** (3.22-5.21)	***8.34** (6.57-10.60)
23F	IgG	**0.02** (0.02-0.04)	***0.19** (0.10-0.36)	***0.33** (0.19-0.55)	***2.26** (1.35-3.78)
	IgA	**0.01** (0.01-0.01)	***0.10** (0.07-0.16)	***0.04** (0.03-0.05)	***0.28** (0.18-0.45)
	IgM	**0.48** (0.38-0.59)	***1.16** (0.88-1.52)	***1.11** (0.87-1.42)	***2.07** (1.59-2.70)

Table 6.3 Geometric mean concentration of anti-pneumococcal antibodies in the serum of 12 month old toddlers following two consecutive doses (two months apart), of Pnc7 administered on day 0 (12 months of age), and day 56 (14 months of age).
Serum was collected prior to and 6/7 days after each dose and was then stored at -80°C. Pneumococcal capsular polysaccharide antibody (IgG. IgA and IgM), were quantified by ELISA, specific for serotypes 4, 14 and 23F. Serum samples were pre-adsorbed with CwPS and serotype 22F polysaccharide. Titres were obtained in μg/ml and the data then expressed and the GMC of IgG, IgA or IgM (95% confidence interval in parenthesis). *** p=<0.0001, significant rise in GMC above baseline following the first dose of conjugate at day 0 and the second dose at day 56.

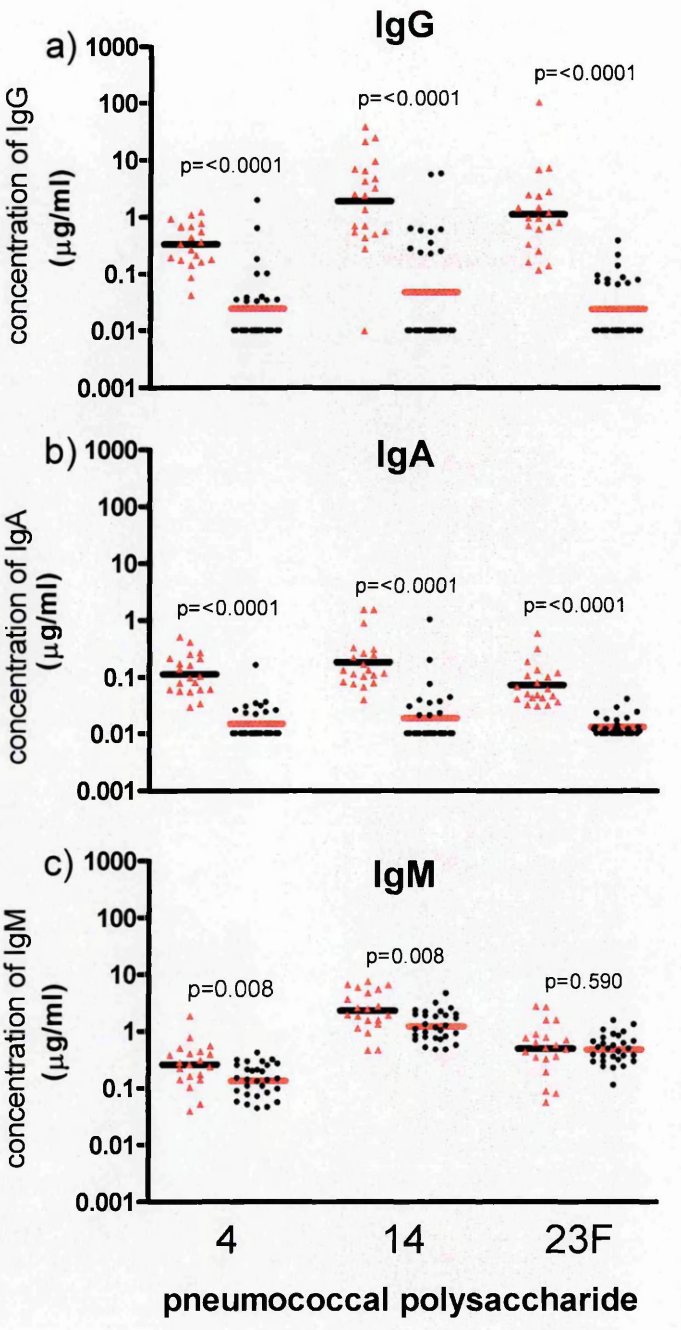


Figure 6.7 Baseline serum IgG (a), IgA (b), and IgM (c) antibody levels in adults (n=20, red triangles), and 12-month old toddlers (n=23, black circles).
Antibody levels against pneumococcal capsular polysaccharides from serotypes 4, 14 and 23F were quantified by ELISA. The data represent the antibody concentration in µg/ml and the bar shows the GMC.

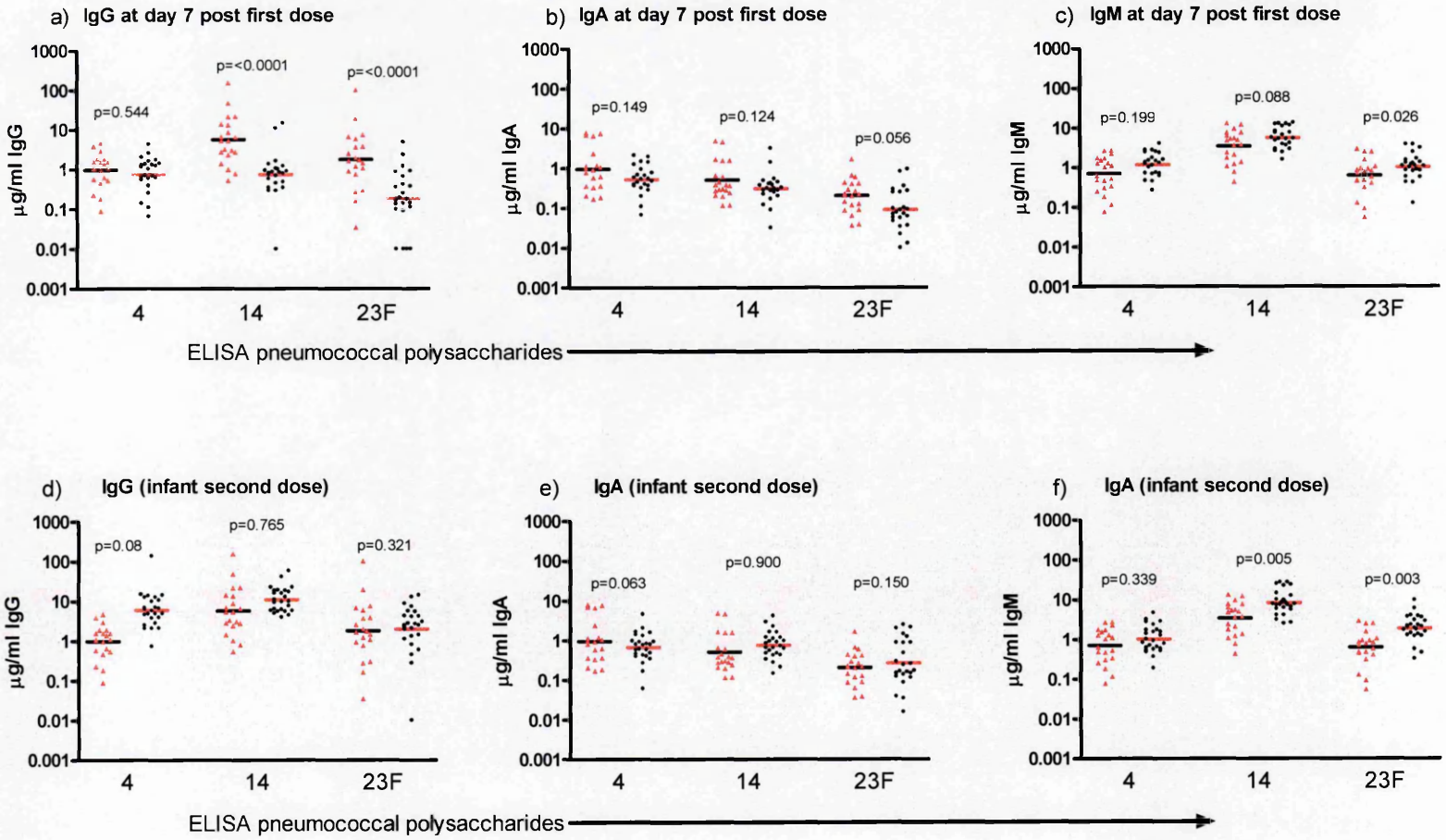


Figure 6.8 Comparison of serum IgG, IgA and IgM antibody levels in adults (n=20, red triangles), and 12-month old toddlers (n=23, black circles). The data in figures (a-c) are seven days after the primary dose of Pnc7 in adults and toddlers. The data in figures (d-f) compares antibody levels seen 7 days after the primary dose in adults versus 7 days after the second dose in toddlers. Antibody levels against the pneumococcal polysaccharides from serotypes 4, 14 and 23F were quantified by ELISA. The data represent the concentration of serum antibody and the bar shows the GMC.

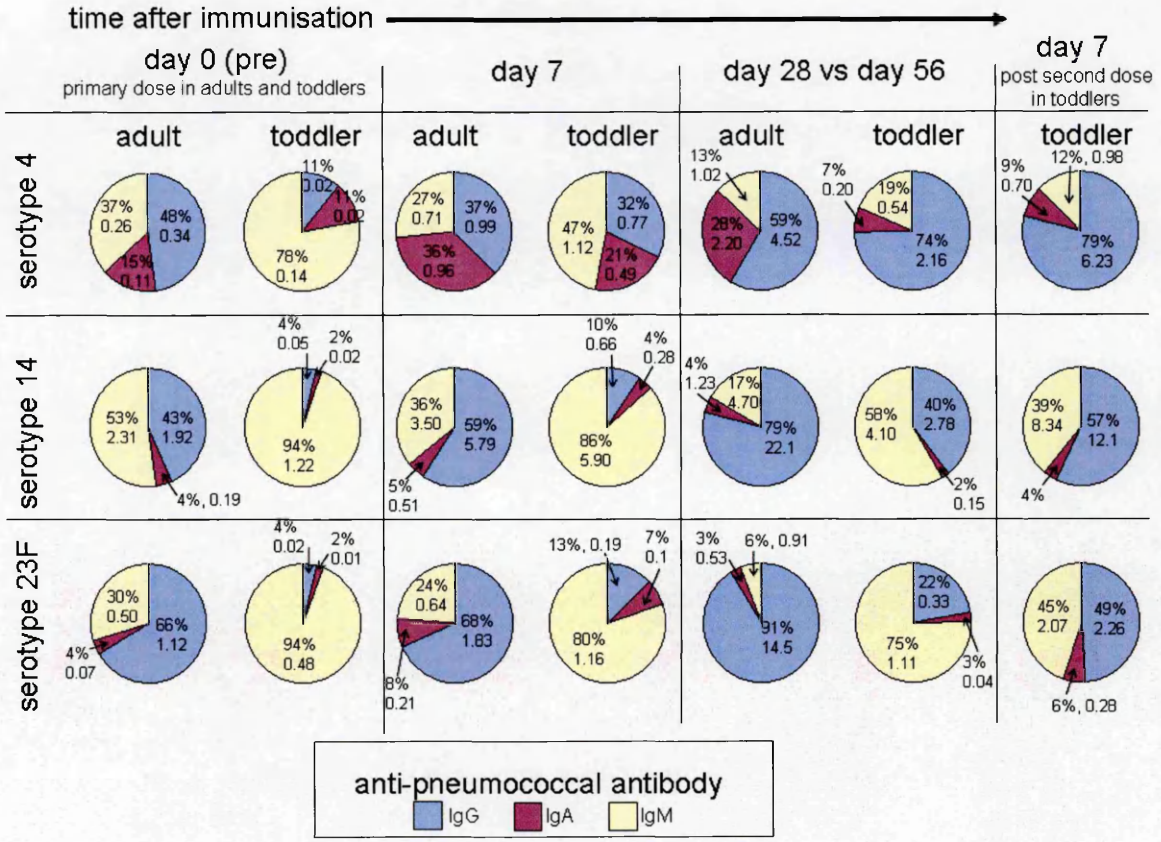


Figure 6.9 The contribution of IgG, IgA and IgM to the anti-pneumococcal polysaccharide response prior to and following immunisation of adults and 12 month old toddlers with Pnc7.

Antibody levels were measured in serum taken prior to and 7 days after primary immunisation. Serum was also obtained from adults on day 28 post primary immunisation and from toddlers prior to receiving the second dose of Pnc7 on day 56 and 7 days after the second dose. The data are shown for antibodies against serotype 4, 14 and 23F and the pie charts show the percentage contribution of each isotype to the over all immunoglobulin response and also the actual GMC antibody concentration.

6.7.10 The effect of age on peripheral blood B cell subset proliferation following immunisation with a single dose of Pnc7

The percentage of $CD19^+CD27^+$ (memory), $CD19^+CD27^-$ (naïve), IgM^+IgD^+, IgM^+only and IgG^+only B cells undergoing >4 cell divisions was quantified, as previously described. This proliferative capacity was then compared between the young and elderly adults for each of the *in vitro* culture conditions (SAC+CpG+PWM versus SAC+IL-2 (figure 6.10). In all cases the percentage of B cells undergoing >4 cell divisions in the SAC+IL-2 system was lower than in the SAC+CpG+PWM system.

Immunisation with a single dose of Pnc7 vaccine affected the percentage of B cells undergoing >4 cell divisons if the B cells expressed an IgM^+IgD^+ or IgG^+ phenotype. Prior to immunisation the percentage of IgM+IgD+ B cells that had undergone >4 cell divisions was lower in the elderly than in the young adult population following stimulation with SCP or SI. After immunisation this difference in proliferation remained for IgM+IgD+ B cells stimulated with SCP but there was no difference in the age groups when SI was used. Also following immunisation there were fewer IgG+ B cell that had achieved >4 cell divisions in the elderly group following SI stimulation of PBMCs.

There were significantly fewer memory B cells ($CD19^+CD27^+$) proliferating in response to *in vitro* stimulation with SAC+CpG+PWM (blue) at day 0 and at day 28 in the elderly (hatched blue), compared to the young adults (plain blue) in fig.6.10a. However there was no effect of age on memory B cell proliferation when PBMCs were stimulated with SAC+IL-2 (red). There was no effect of age or *in vitro* stimulant on the proliferation of the naïve ($CD19^+CD27^-$) B cell subset at day 0 or day 28 (fig.6.10b).

The IgM^+IgD^+ B cell subset (fig.6.10c), showed poorer proliferative capacity in the elderly than in the young adults. This was seen in response to both SCP and SI at day 0 but following immunisation the poorer proliferative response was only observed in response to *in vitro* stimulation with SCP. IgM^+only B cells proliferated equally as well in both age groups and in

both culture systems while IgG^+ B cells proliferated less well in the SI system in the elderly than in the young adults.

Therefore there appears to be an age related deficiency in the capacity of $CD19^+CD27^+$ and IgM^+IgD^+ B cells to proliferate in response to *in vitro* stimulation with SCP.

6.7.11 The effect of age and the type of vaccine on polyclonal, *in vitro* stimulation of peripheral blood B cell subsets.

The data in figure 6.11 shows the B cell proliferation of PBMCs obtained on day 0 and day 28 following immunisation with the heptavalent conjugate vaccine (Pnc7) or 23-valent polysaccharide vaccine (23PsV). As already seen in figure 6.10, the percentage of proliferating B cells was significantly reduced in the SI culture system compared to the SCP system. Aside from this difference there was no effect of vaccine or age on the *in vitro* proliferative response of the B cell subsets.

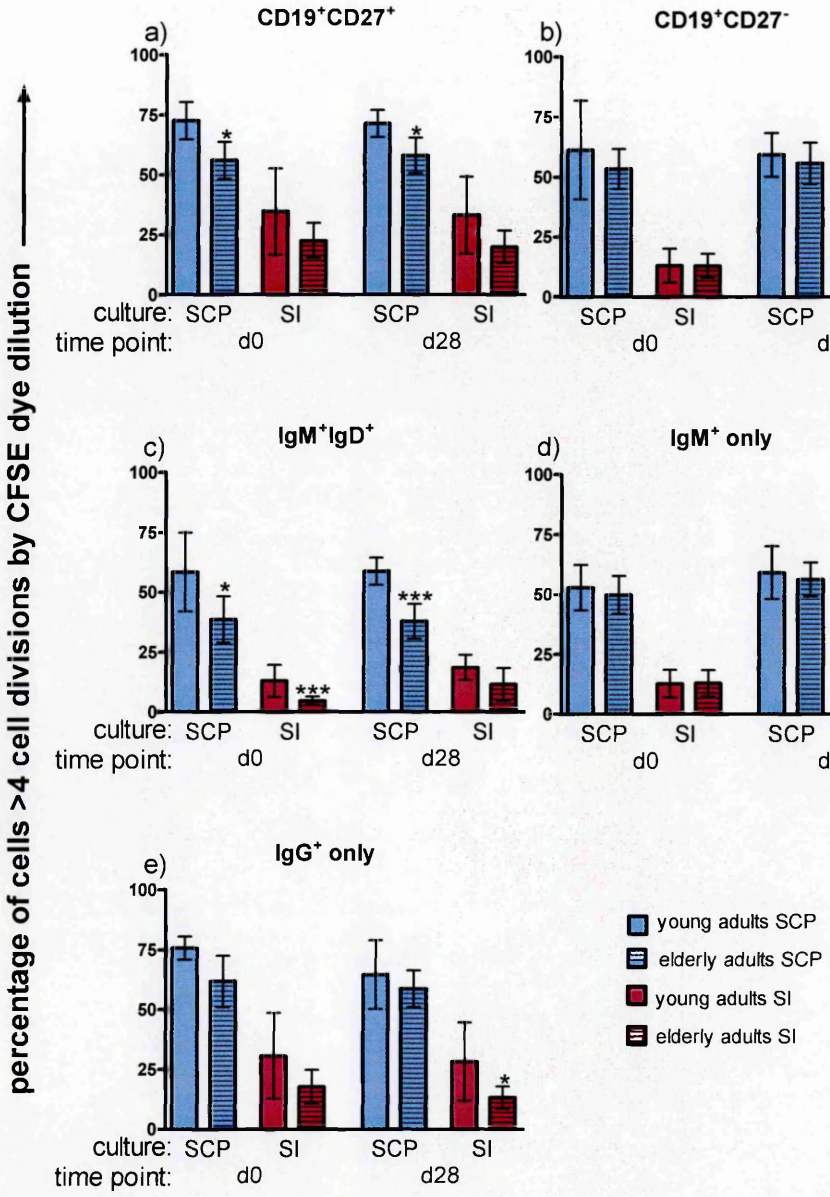


Figure 6.10 The effect of age on the *in vitro* stimulation of peripheral blood B cells from young and elderly adults.

PBMCs were isolated prior to and 28 days after a single dose of Pnc7 was given to a group of young adults (n=10, plain columns) and elderly adults (n=18, hatched columns). The PBMCs were then labelled with CFSE and stimulated for 5 days with SAC+CpG+PWM (SCP, blue) or SAC+IL-2 (SI, red). After 5 days the cells were harvested and labelled for the surface expression of: a) $CD19^+CD27^+$, b) $CD19^+CD27^-$, c) IgM^+IgD^+, d) IgM^+ only and e) IgG^+ only. The data represent the mean percentage of cells within the specified populations that had achieved >4 cell divisions during the 5 day culture. The bars are the standard error. (*p=<0.05, ***p=<0.01).

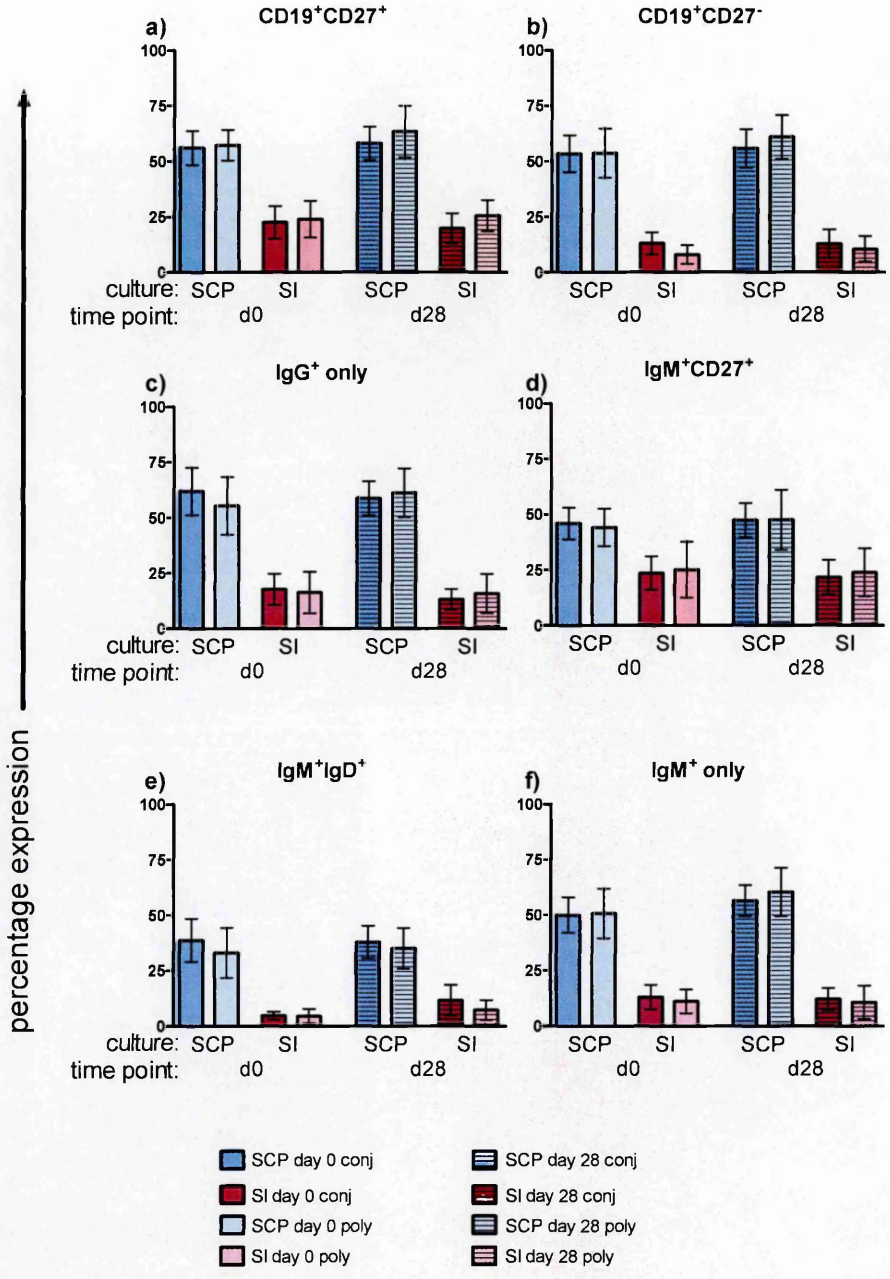


Figure 6.11 The effect of *in vitro* stimulation on the proliferation of B cells isolated from the peripheral blood of elderly adults.
PBMCs were isolated on day 0 (d0, plain columns) and 28 days after (d28, hatched columns) immunisation. The elderly adults were given either a single dose of Pnc7 (n=18, dark blue, red) or 23PnV (n=9, pale blue, pink). The PBMCs were labelled with CFSE and cultured for 5 days with SCP (dark and pale blue) or SI (red and pink). After 5 days the cells were harvested and labelled for the surface expression of: a) $CD19^+CD27^+$, b) $CD19^+CD27^-$, c) IgM^+IgD^+, d) IgM^+ only and e) IgG^+ only. The data represent the mean percentage of cells within the specified populations that had achieved >4 cell divisions during the 5 day culture. The bars are the standard error.

6.7.12 *In vitro* stimulation of PBMCs with SCP or SI and the detection of IgG-AFC at baseline.

Prior to immunisation (d0), there was no significant difference in the frequency of total-IgG-AFC in the conjugate vaccine group (fig.6.12a), while in the polysaccharide vaccine group stimulation with SCP induce a significantly higher number of total-IgG-AFC. There was a slightly higher frequency of tetanus toxoid specific IgG-AFC following *in vitro* culture with SCP than with SI in the conjugate group while no difference was seen in the polysaccharide group (fig.6.12b). *In vitro* stimulation with SCP and SI made no significant difference to the detection of diphtheria toxoid specific IgG-AFC at d0 in either vaccine group (fig.6.12c).

The frequency of serotype 4, 14 and 23F specific IgG-AFC at d0 was slightly higher following *in vitro* stimulation of PBMCs with SCP ($p=<0.05$) in the conjugate group (fig.6.12d). The only such difference seen in the polysaccharide vaccine group was in IgG-AFC specific for serotype 23F which were of higher frequency following *in vitro* stimulation with SCP, $p=<0.05$ (fig.6.12e).

6.7.13 *In vitro* stimulation of PBMCs with SCP or SI and the detection of IgG-AFC 28 days after immunisation.

Twenty-eight days after immunisation with the pneumcoccal conjugate vaccine (Pnc7) the frequency of IgG-AFC specific for total-IgG (fig.6.12a), tetanus toxoid (fig.6.12b), diphtheria toxoid (fig.6.12c) and serotypes 4, 14 and 23F (fig.6.12d. $p=<0.001$) was significantly higher following *in vitro* stimulation of PBMCs with SCP than with SI.

There was no difference to the frequency of diphtheria toxoid specific IgG-AFC (fig.6.12c) between SCP and SI stimulation following immunisation with the polysaccharide vaccine. However, the frequency of IgG-AFC specific for total-IgG and tetanus toxoid (fig.6.12a-b) was significantly higher following stimulation of PBMCs with SCP than with SI and this was also true for serotype 14 polysaccharide (fig.6.12e).

Immunisation with the pneumococcal conjugate vaccine followed by *in vitro* stimulation of day 28 PBMCs with SAC+CpG+PWM was the only combination that revealed a significant rise in the frequency of memory B cell derived IgG-AFC specific for diphtheria toxoid and serotypes 4, 14 and 23F (fig. 6.12c-d). SAC+IL-2 stimulation did not reveal any increase in memory cell frequency from d0 to d28. Following immunisation with the polysaccharide vaccine *in vitro* stimulation with SCP or SI failed to show any rise in memory B cell derived IgG-AFC frequency. PBMC stimulation with SAC+CpG+PWM revealed a significant higher frequency of IgG-AFC induced by the conjugate vaccine rather than the polysaccharide vaccine, except for serotype 14. With SAC+IL-2 the only difference between the vaccines was a higher diphtheria response in the conjugate group (fig.6.12c).

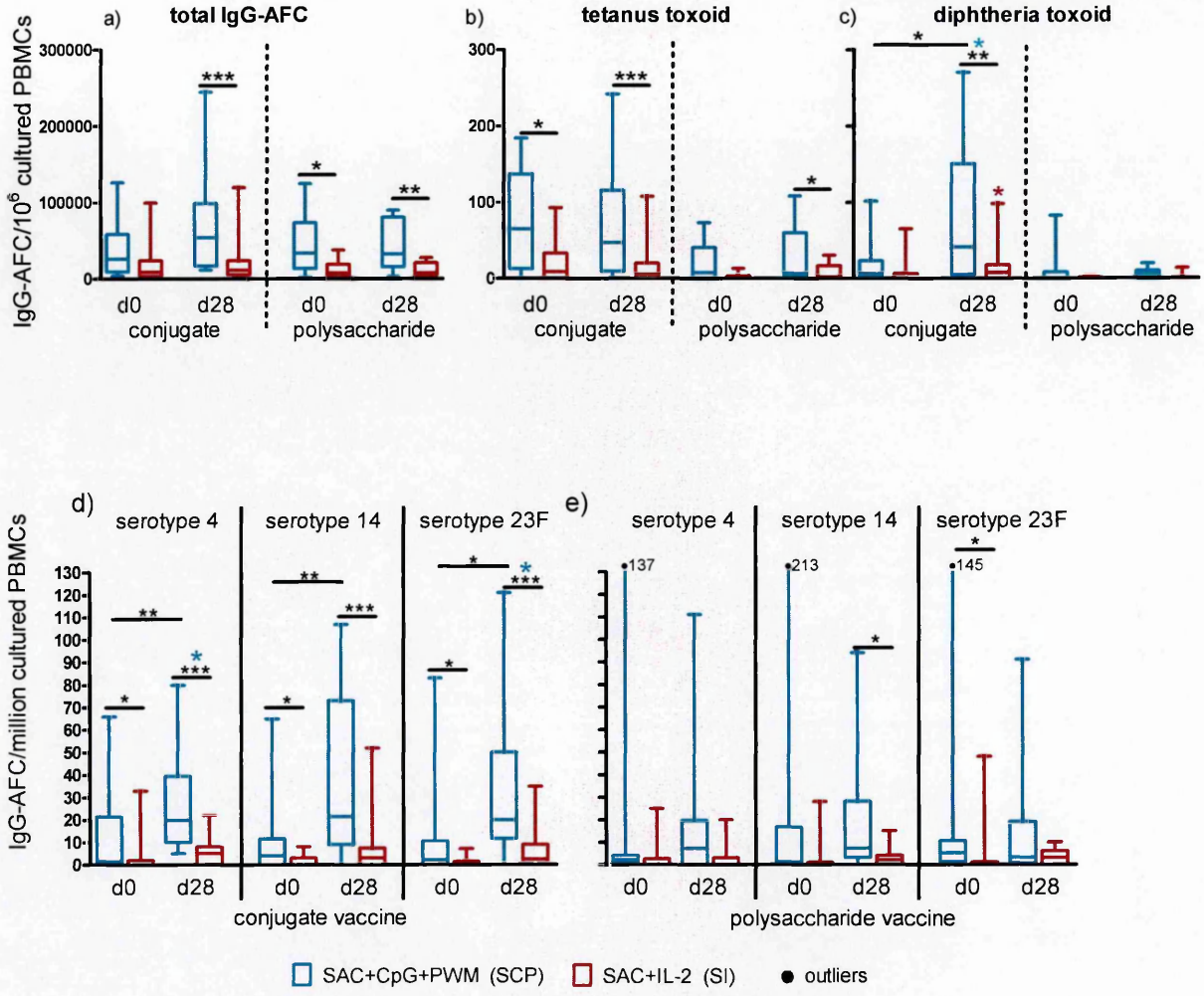


Figure 6.12 The effect of *in vitro* stimulation on the IgG-AFC response to a pneumococcal conjugate or plain polysaccharide vaccine in the elderly.

PBMCs were isolated from elderly adults prior to (d0) and again 28 after (d28) a single dose of conjugate vaccine (n=18), or polysaccharide vaccine (n=9). The PBMCs were cultured for 5 days with SAC+CpG+PWM (blue) or SAC+IL-2 (red). After 5 days the cells were harvested and seeded into antigen specific ELISpot plates coated with; (a) anti-Ig, (b) tetanus toxoid, (c) diphtheria toxoid, (d+e) serotype 4, 14 and 23F polysaccharides. The boxes represent the 25th and 75th percentile, the whiskers show the outliers and the black dots are the extreme outliers. The central bar is the median IgG-AFC/10^6 cultured PBMCs. Wilcoxon paired, non-parametric test was used to compare IgG-AFC frequencies between the SCP and SI cultures within the conjugate vaccine group and the polysaccharide group (*). The same analysis was used to compare d0 with d28 within each vaccine group (*). Comparison of IgG-AFC frequency between vaccine groups was made using the Mann Whitney U test for un-paired, non-parametric data(*). (*p=<0.05. **p=<0.01, ***p=<0.001).

6.8 Discussion

This comparative study of the immune response to a pneumococcal conjugate vaccine in young and elderly adults and 12 month old toddlers and has shown that individuals of all ages responded well to a single dose of the conjugate vaccine. Young and elderly adults responded equally well while the response in the toddlers was significantly lower. This observation was true with respect to spontaneously secreting IgG-AFC and *in vitro* stimulated IgG-AFC aswell as with serum IgG, IgA and IgM antibody levels in young adults compared to toddlers. The difference in the magnitude of the B cell response may explain the short duration of antibody production and vaccine effectiveness at a population level following conjugate vaccine immunisation, in early childhood.

The second dose of vaccine in infants, 2 months after the primary dose, brought their memory B cell derived AFC and antibody responses to a similar level as seen in the young adults after one dose, suggesting that more doses of glycoconjugate vaccines may be needed in the 12 month old age group to generate the same size B cell memory pool as seen in young adults.

The responding B cells may be important both for sustained antibody responses and the rapid rise in antibody following re-exposure to the antigen during invasive disease. Indeed, booster doses of conjugate vaccines are now widely used at 12-15 months of age and induce memory responses with rapid elevations in antibody concentration. These rises in antibody concentration are seen even when pre-booster antibody levels are negligible.

However, despite generating similar levels of memory B cell derived AFC, a second dose of Pnc7 did not alter the difference in magnitude of the *ex vivo* plasma cell response in the infants which remained significantly lower than that seen in the adults one week after a single dose. Although not proven, it is likely that protection against rapidly invasive micro-organisms is provided by maintenance of protective serum antibody levels and by the rapidity of antibody rise following infection or booster dose of vaccine, (provided by the *ex vivo* plasma cell response). Also it is

possible that the *ex vivo* plasma cell response is generated from a different B-cell subset, such as the marginal zone B cells that are fewer in number in infants compared to older age groups(253). In order to better understand the mechanism involved in maintaining protective immunity further studies of the B cell phenotypes induced by immunisation and differences, between the B-cells isolated from infants and from young adults needs to be undertaken (252, 318, 494).

6.8.1 Baseline immunity prior to a single dose of Pnc7

Prior to immunisation, all of the young adults in the study had IgG-producing memory B-cells specific for the capsular polysaccharides of serotypes 4, 14 and 23F, while in the elderly and the 12 month old toddlers there was little if any detectable memory B cell derived IgG-AFC following *in vitro* stimulation of the B cells with SAC+CpG+PWM. Similarly, the young adults had pre-existing serum IgG above protective levels for all three serotypes tested while IgG was almost undetectable in the toddlers. On the other hand the levels of IgM were quite similar in the toddlers and young adults (table 6.2 and figure 6.7c).

The absence of naturally acquired, polysaccharide specific B-cell memory in infants prior to immunisation with Pnc7 may relate to differences in nasopharyngeal carriage of pneumococci or immaturity of the immune system when compared with adults. However, most infants at 12 months of age are known to have experienced some nasopharyngeal carriage of pneumococci (59). In one Oxford study vaccine type pneumococci were isolated from 38% of infants and vaccine related types (6A, 19A, 23A), were isolated from 13% of infants at 12 months of age (397), and other studies have shown that more than 80% of children will have carried pneumococci by 1 year of age, with even higher rates in resource poor settings (79, 495, 496). These observations suggest that exposure of infants to pneumococcal carriage in the first year of life may fail to induce IgG memory in these infants.

It may be that repeated exposure to the capsular antigens is the necessary requirement to maintain detectable levels of IgG memory and that this immunological experience is only possible over

some years (497). Interestingly, in this study adults had higher levels of naturally acquired IgG to the more commonly carried serotype-14 and 23F than to the rarely detected serotype-4 (58, 59, 92).

The elderly adults may also have lower rates of pneumococcal carriage than the younger adults since carriage decreases with age (489). There is also a decrease in serotype specific IgG with age, although 99% of >65's had antibody against the cross-reactive cell wall polysaccharide (Cw-PS) despite lacking capsule specific antibody (491).

Infants had higher levels of IgM to these serotypes, but no IgG. It is known that MZB are involved in the response to polysaccharide antigens and that MZB predominantly secrete IgM with some IgG (144, 252, 266, 267, 315, 323). Therefore it is possible that natural anti-polysaccharide immunity in young infants may be mediated entirely by this B-cell subset while there may be a greater role for follicular B cells in adults, and that other factors are required to induce a switch from IgM to IgG/A as is seen in the adults, such as cytokines secreted by by-stander activated T cells (452).

Several other factors must be considered that may contribute to the apparent immaturity of the infant immune response and the inability to maintain an IgG-memory response. Infants may lack appropriate bone marrow niches for long-lived plasma cell survival (142, 350, 356), and their splenic structure lacks some adult functional features such as $CD27^+$ B-cells(317) and CD21 expression(494, 498, 499). The low frequency of $CD27^+$ memory B-cells may just be a result of fewer antigen exposures than in adults. Low expression of CD21 (complement receptor CR2) reduces the strength of signaling via BCR-co-receptor complex(316, 499, 500), therefore antigen presentation in toddlers is less efficient than in adults. IgM is a potent activator of complement, but a lack of surface CD21 on infant MZB might reduce the strength of the signal received by the B-cell.

There was no significant difference between the age groups in frequency of baseline diphtheria specific IgG-AFC. More than 80% of 1 year old toddlers had pre-existing memory to the protein

antigens, presumably induced by 3 dose priming with routine immunisations during the first 6 months of life but we do not have data to provide a comparison with a conjugate vaccine vaccine given in the same schedule.

6.8.2 Response to the first dose of Pnc7 conjugate

It was proposed that the cells detected *ex vivo*, in the first week following immunisation (figure 6.2) are likely to be short-lived mature plasma cells generated from extra-follicular differentiation of B-cell subsets such as MZB and B1 B-cells (127, 263, 398). These mature, antigen specific plasma cells disappear from peripheral blood by day 9 (432, 501), but non-secreting plasmablasts may still be circulating (385).

In vitro stimulation of the PBMCs taken at these same time points drives proliferation and differentiation of plasmablasts and memory B cells *in vitro* to AFC which can be detected in the Elispot assay (350). It is presumed that the memory response, which involves germinal centre formation, consists of newly generated memory cells and precursors of long-lived plasma cells (263, 354). By day 15, the cells present in the *in vitro* stimulation Elispot may include; i) mature plasma cells that probably do not survive the culture system after 5 days and so are unlikely to contribute to the frequency of AFC detected in the Elispot, ii) memory B-cells mobilized, following immunisation, to re-circulate via the peripheral blood and back to the lymphoid tissues and, iii) long-lived plasma cell pre-cursors migrating to the bone marrow where they take up residence for long-term antibody production (145, 338, 350, 357). Either of these latter two subsets may contribute to the measured Elispot response. Migration to the bone marrow or re-circulation back to the lymphoid tissues could explain the subsequent waning of the memory response by day 28 back to baseline levels. Long-lived plasma cells are thought to be the dominant cell type in circulation following antigen challenge at day 14-15 following immunisation, but are not necessarily mature enough to secrete antibody without stimulation until

they reach bone marrow niches (263). This could explain why, if present, such cells disappear from the *ex vivo* assay and remain elevated in the *in vitro* assay.

6.8.3 B cell subset proliferation following a single dose of Pnc7

Because of the uncertainty of the B cell type being detected in the *in vitro* stimulated ELISpot a comparison was made of the B cell proliferation in the young with that in elderly adults. PBMCs were cultured with SAC+CpG+PWM or SAC+IL-2 for 5 days. The *in vitro* proliferation of the different B cell subsets, before and after immunisation, was then compared. The elderly adults demonstrated less proliferation of memory (CD27+CD19+) B cells when PBMCs were cultured with SCP. This was the same before and after immunisation. There was no such difference when PBMCs were stimulated with SAC+IL-2. There were also fewer IgM+IgD+ B cells at the same time point under the same conditions. However there was also reduced proliferation of this subset in the SI group prior to immunisation. There were no differences in the the proliferation of IgM only B cells , while IgG only B cells differed at day 28 after stimulation with SAC+IL-2. This difference in IgG only B cells suggests that there were fewer recently activated memory B cells in the circulation in the elderly than in the young adults (fig.6.10e). There was no difference following stimulation with SCP which shows that T cell help and CpG-TLR-9 were required in both age groups for maximum IgG^+ B cell proliferation. Together, these results suggest that there is a deficit in the CD27+IgM+IgD+ B cell population in the elderly population. This is the phenotype of MZB that are implicated as having a role in anti-polysaccharide responses and also decrease in frequency with age (figure 1.4 in the introduction) (123, 251, 252, 273, 301, 502).

Another reason for age related differences in response to the conjugate may result from nasopharyngeal carriage of pneumococci, as demonstrated in mouse models (240, 497). Continuing exposure to pneumococcus over many years may enhance the adult immune response to Pnc7(497) while in 12-month old infants this effect is minimal in comparison, resulting in the lower AFC frequencies observed and an antibody response dominated by IgM production. This is

also the case in the elderly who had higher baseline memory cell frequency than the toddlers but less than the young adults. This link between the effectiveness of conjugate immunisation and carriage was recently suggested in the UK where lack of exposure to Hib carriage led to increased disease among children given a priming course of immunisations but no booster (478).

The difference in magnitude of the response to the polysaccharide antigens between adults and toddlers does not correspond to differences in the response to the carrier protein. Indeed there was no significant difference in the frequency of diphtheria IgG-AFC (diphtheria toxoid contains B cell epitopes included in the CRM197 carrier protein of Pnc7) between the age groups, prior to or following immunisation.

The anti-polysaccharide antibody response to a single dose of conjugate vaccine was dominated by IgM, despite the recruitment of T-cell help by the carrier protein in the toddler group. It has previously been shown that antibody to carrier proteins can inhibit anti-capsular responses following glycoconjugate immunisation where there is a high anti-carrier protein antibody concentration present(149, 190, 503, 504). This is either because serum antibody mops up antigen, targeting it for macrophage engulfment before a B-cell is activated or that epitope specific antibody blocks B-cell binding. The diphtheria memory-AFC response in the infants appeared to reach a plateau, and the *ex vivo* plasma cell response showed a slight decrease on re-immunisation. These results may imply that antibody concentration was involved in regulation of the magnitude of the response.

6.8.4 Response to the second Pnc7 dose in infants

Re-immunisation of the infants at 14-months of age failed to generate increased frequencies of *ex vivo* AFC, when compared with the response 2 months earlier. It has previously been shown that re-immunisation of adults with polysaccharide does not boost the frequency of spontaneously

secreting plasma cells (108), but the effect of a second immunisation with a conjugate vaccine is not certain and is age and serotype dependent in adults, working less well in the elderly than in young adults(410). It has also been suggested that the secondary response comprises mainly plasmablasts, leaving the lymphoid tissues and migrating to the bone marrow. These cells are not necessarily secreting antibody until they receive further stimulation. Therefore they may also be detected in the *in vitro* system rather than the *ex vivo* system. In contrast the memory response generated by the first dose of conjugate was boosted following the second immunisation in the infants.

All of these data suggest that the short-lived plasma cell response is highly regulated and is probably involved in controlling infection before the memory response (which includes long-lived plasma cells secreting high affinity IgG antibody), can develop. It also suggests that the *ex vivo* and memory responses to immunisation may be independent (505). In relation to this, it has previously been shown that infants immunised with a MenC conjugate at 4 years of age, who generated no serum bactericidal response were still capable of generating a high magnitude response when boosted 2 years later. These data suggest that memory was laid down even in the absence of an *ex vivo* (in this case serum antibody), response, providing further evidence of separate mechanisms for memory versus short-term antibody responses (506).

The frequency of diphtheria specific memory IgG-AFC appeared to reach a plateau following the first dose of conjugate, declining slightly during the two month period before re-immunisation and was not boosted following re-immunisation. An inverse relationship has been described between AFC frequency and number of does of diphtheria vaccine in adults (372). The frequencies of diphtheria specific memory cells in toddlers in the study reported in this chapter were equivalent to those seen in adults after a single dose of Pnc7. Obunkhanych *et al* 2006 (507) have recently demonstrated that serum IgG levels can regulate the responsiveness of B-cells to antigen stimulation. Infants already have immunity to diphtheria and it may be that pre-existing,

high affinity antibody limits the magnitude of the diphtheria response following Pnc7 re-immunisation (508, 509).

6.8.5 The antibody response following the first and second dose of Pnc7

The antibody concentration following the first dose of Pnc7 was similar to that seen in 2-5 year old children primed by 23PsV (178). Therefore, any pre-existing carrier specific memory or pre-exposure to nasopharyngeal carriage did not seem to affect the response to the first dose given at 12 months of age. Even so the data for infants (table 6.2), did show evidence of class switching in the week following the second dose of conjugate. This was particularly evident for serotype-4 that was predominantly IgG mediated by day 63, while the responses to serotypes-14 and 23F were still a mixture of IgG and IgM. This suggests that even though the first dose of vaccine induced antibody that was mainly of IgM, memory was being generated to enable a more rapid appearance of IgG following the second dose. The changing antibody isotypes are more clearly visualised in figure 6.11.

The mixed IgG/IgM response may be of benefit in protection against IPD. IgM protects against blood borne infections and the higher IgM levels induced following immunisation with serotypes-14 and 23F may reflect their reduced invasiveness compared to serotype-4 that induced little IgM in response to immunisation.

In this study differences were found in the IgA concentration generated in response to the different serotypes. IgA is transported to mucosal surfaces where it can block adhesion of bacteria to epithelial cells. It also functions as an opsonin, leading to uptake and killing of bacteria by macrophages and neutrophils via the FcαR and Fcα/μR(41). Serotype-23F induced the lowest IgA response. This might mean it is more able to adhere to mucosal surfaces and be less rapidly cleared from the nasopharynx than serotypes-14 and 4. In previous studies on carriage and disease, serotype-23F has been the most frequently detected nasopharyngeal isolate (58, 59, 92).

Of the three serotypes investigated serotype-4 induced the best IgA response following the primary immunisation. This serotype is almost never detected in carriage studies, a characteristic that may be related to its propensity to stimulate IgA production (58, 59, 92). The infrequency of serotype-4 carriage may explain the similarity in response to this serotype between adults and infants.

6.8.6 The difference in B cell subsets recruited following immunisation with Pnc7 or 23PnV in the elderly.

Since there was such difference in the baseline memory responses between the age groups, but only for the capsular polysaccharides, it became important to determine whether there were age related differences in B cell subset proliferation and differentiation into IgG-AFC. The data in figure 6.6 show that prior to immunisation there was a significantly lower frequency of IgG-AFC in elderly adult peripheral blood than was seen in the peripheral blood of young adults. Following immunisation with a single dose of Pnc7 similar frequencies of polysaccharide and protein specific IgG-AFC were induced in both young and elderly adults (figure 6.5b+c). These data were obtained using *in vitro* stimulation of peripheral blood with SAC+CpG+PWM. By using this system of stimulation it has also been possible to show that the elderly volunteers responded more robustly to the conjugate vaccine in comparison to the polysaccharide vaccine but this was not as apparent from the B cell proliferation studies.

This study has shown is that SAC+CpG+PWM stimulation of PBMCs significantly enhanced the IgG-AFC frequency detected in response to the conjugate and polysaccharide vaccine and it was significantly higher than after SAC+IL-2 stimulation. The IgG-AFC response following Pnc7 immunisation increased significantly above baseline to the polysaccharide and carrier protein antigens when PBMCs were pre stimulated with SAC+CpG+PWM. Pre-stimulation of PBMCs with SAC+IL-2 showed no difference in IgG-AFC following immunisation with the conjugate or

polysaccharide vaccine, infact the responses were identical, to both vaccines in the SAC+IL-2 group, except for the diphtheria response.

Thus the difference between these two systems has now been shown to result in different outcomes of response to immunisation with Pnc7 versus 23PsV, with above baseline IgG-AFC frequencies detectable at day 28 in the SCP system but not in the SI system (fig.6.12c-d).

As discussed in chapter 5, SAC+IL-2 stimulates B cell proliferation by a similar mechanism to specific antigen. It therefore only induces AFC formation from *in vivo* activated B cells. These cells have returned to pre-immunisation frequencies by day 28 so it looks as though no response was made when this combination of stimulants is used. SAC+CpG+PWM is able to activate resting B cells and also long-lived plasma cells that are not mature enough to secrete antibody without stimulation until they reach bone marrow niches (263). These cells are present for up to 6 weeks post immunisation and so the response remains elevated above baseline by day 28. Diphtheria specific B cells are able to recruit antigen specific T cell help and therefore there is still an elevated response in the SAC+IL-2 group. The polysaccharide vaccine only induces short term B cell activation *in vivo* with no germinal centre formation, and therefore neither culture system is able to induce AFC formation as the cells required are not in circulation at day 28. It might be expected that the conjugate and polysaccharide vaccine responses would be similar at day 7 with either culture system and would be elevated above baseline since the B cells at that time point would be antigen activated and it was shown in chapter 5 that SCP and SI induced identical responses on days 6-7 post immunisation.

6.9 Conclusion

This study of the immune response to immunisation with pneumococcal vaccines has shown that the human toddlers require at least two priming doses of Pnc7 to achieve memory B cell and IgG antibody levels of similar magnitude as those found in young adults. Despite improved memory

B cell and serum IgG concentrations following a second dose of Pnc7, the generation of *ex vivo* plasma cell remained significantly reduced in toddlers compared to young adults. The peak in spontaneously secreting AFC may well be derived from MZB responses that occur early in the immune response and explains why they are lower in infants. It would be interesting to make the comparison in the elderly if the frequency of MZB is falling with old age. It would be expected that young adults would have the highest *ex vivo* response, followed by the elderly and then the toddlers depending on the extremes of the age differences.

Lower frequencies of polysaccharide-responsive B-cell subpopulations, absence of the splenic marginal zone, impaired maintenance of long-lived plasma cells, and age-dependent differences in polysaccharide immune responsiveness may all contribute to poor maintenance of immune responses to glycoconjugate vaccines in infants and explain the higher risk from invasive disease in this age group.

It is important to choose the time point and *in vitro* antigen stimulation carefully otherwise it may appear that individuals are not responding to immunisation or that there was no memory response. Continued exposure through nasopharyngeal carriage may help maintain adult memory B cell levels and booster doses of vaccine might overcome the lack of sustained immunity in young children by increasing the size of the memory B cell pool to young adult levels.

Chapter 7: Discussion.

7.1 Summary

Invasive pneumococcal disease remains one of the most significant global causes of childhood mortality, particularly in children under 2 years of age(37, 62-65). The heptavalent pneumococcal polysaccharide-CRM197 conjugate vaccine (Pnc7) has been introduced to target the major disease causing serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) in childhood in many countries.

The effectiveness of this vaccine has been clearly demonstrated in the United States, where there has been a 93% reduction in IPD in the <5 year olds and also a reduction in carriage(152, 157, 158, 510-512). Herd immunity resulting from a decrease in nasopharyngeal carriage in young children (who are the reservoir for transmission) impacted on the rates of adult disease, reducing the rates of vaccine type disease cases(152, 157, 158, 510-512).

All age groups immunised so far in various studies (table 1.2) have demonstrated good serum IgG antibody responses as well as effective boosting with plain polysaccharide after priming with Pnc7. The purpose of this thesis was to determine whether it was possible to detect pre-existing immune memory B cells in the peripheral blood of toddlers and adults and whether the magnitude of the B cell response to Pnc7 immunisation reflected the subsequent antibody response.

The major findings of this thesis have been to define the kinetics of the plasma cell and memory B cell responses in adults following immunisation with Pnc7 (Chapter 4). This enabled inter age group comparisons of the B cell responses to be made between 12 month old toddlers, young adults and the elderly (Chapter 6). The phenotypes of B cell populations in peripheral blood,

following immunisation, were identified. The effect of depleting some of the B cell subsets on IgG-AFC frequency was also determined. The effect of *in vitro* stimulation of peripheral blood B cells was also investigated along with how this altered the perceived memory B cell response following immunisation (Chapter 5).

7.2 Antigen specific plasma cells and memory B cells were detected in toddler peripheral blood.

The first aim of this thesis was to determine whether it was possible to detect polysaccharide specific B cells in peripheral blood of toddlers.

A single dose of Pnc7 induced a significant rise in dip specific IgG plasma cell frequency by days 9-11 post immunisation with a similar, but none significant trend in response to the polysaccharides of serotype 4 and 14 but not serotype 23F. Isolation of cells at day 7 (Chapter 6, fig 6.1) showed that the toddlers made a robust plasma cell response to all antigens tested. Previous toddler studies (age <24 months), have quantified the *ex vivo* plasma cell response 7 days after immunisation, finding elevated frequencies of polysaccharide specific IgG-AFC specific for the vaccine given, a quadravalent-mixed carrier protein glycoconjugate (113). However, the IgG plasma cell frequency resembled that seen in adults after polysaccharide immunisation rather than after a dose of conjugate vaccine (113, 383). A second dose of Pnc7 (Chapter 6, fig 6.1), did not alter the magnitude of the plasma cell response.

There is no previous data about memory B cell responses in toddlers. However, it has been possible to show elevation of memory B cell frequency in toddlers in the studies included in this thesis. In this thesis the toddlers immunised with a single dose of Pnc7 demonstrated serotype specific IgG antibody responses similar to those obtained from another UK study in toddlers following a single dose of a 9-valent pneumococcal conjugate vaccine (Pnc9) (175). Our study

found serum IgG specific for serotype 4 and 14 and 23F rose significantly after a single dose when measured on day 7 and day 56 of the response. In a Finish study the serum IgG data obtained from 3 month old infants after a single dose of Pnc7 also showed elevated levels of IgG in response to serotypes 4 and 14 but not to 23F (187).

The generation of memory B cells in our toddler studies appeared to mirror the antibody response although the rise in memory cell frequency was serotype dependent and might be evidence of serotype specific priming (513). Priming by immunisation with conjugate vaccine in the toddler age group has been demonstrated for meningococcal group C (MenC) vaccines. Toddlers receiving a booster immunisation of MenC polysaccharide vaccine mounted rapid rises in serum bactericidal (SBA) activity if previously primed with MenC conjugate vaccine at 12-15 months of age (514). Priming of toddlers with one or two doses of Pnc9 at 12 months of age also primed for enhanced responses to the polysaccharide vaccine one year later (175). This is also seen in the response to the second dose of conjugate in toddlers in this thesis. Antibody responses switched from IgM to IgG and memory B cell frequencies continued to increase. Despite the similarity between the memory B cell and antibody responses to Pnc7 however, there were no significant correlations between cell frequency and IgG concentration. A recent review by Amanna *et al* (515) described a similar lack of correlation between serum IgG and circulating memory B cell frequency in response to tetanus and diphtheria toxoid immunisation and Epstein Bar virus and varicella-zoster virus infection (515).

7.3 Adult peripheral blood B cell responses peaked on days 6-7 after a single or booster dose of Pnc7.

Immunisation of adults with a booster dose of Pnc7 induced a peak in frequency of antigen specific plasma cells on days 6-7, as reported in other studies (Chapter 4 fig.4.1)(107, 108, 111, 516, 517). The response varied in magnitude between individuals and for the different antigens

(Chapter 4 fig.4.2). *In vitro* stimulation of PBMCs with SAC+IL-2 allowed detection of memory B cells which also peaked in frequency at day 7 following immunisation (Chapter 4. fig.4.4). The magnitude and timing of the polysaccharide specific B cell response in adults was the same following a single or booster dose of Pnc7 (Chapter 4 fig.10 and 4.11). A similar observation was seen following repeat immunisation with 23 valent polysaccharide vaccine in adults (108). Carrier protein (diphtheria) specific plasma cell and memory B cell frequency was higher after a single dose than after the booster dose. A previous study of diphtheria toxoid specific memory B cell responses in adult humans, using SAC+IL-2 for *in vitro* stimulation, showed an inverse relationship between number of doses and memory cell frequency (372), thus supporting the results from these studies.

7.4 Phenotype of the plasma cells responding to Pnc7 immunisation of adults.

It is known that MZB and FO B cells fulfil distinct roles in response to infection or immunisation. The phenotype of these cells is well documented but the effect of glycoconjugate immunisation on these B cell subsets is not thoroughly understood. Part of this thesis involved identification of vaccine specific plasma and memory B cells in the peripheral blood.

Initially PBMCs depleted of or enriched for B cells expressing CD27, CD38 or CD20 were used to identify the plasma cells in the peripheral blood following immunisation, and positive ELISpot results were obtained from the $CD27^{+}$, $CD38^{+}$ and $CD20^{+/-}$ fractions. This data suggested the presence of two populations of spontaneously secreting plasma cells in peripheral blood on day 7 after immunisation. They were $CD20^{+}CD27^{+}CD38^{+}$ and $CD20^{-}CD27^{+}CD38^{+}$ with low level surface immunoglobulin expression compared to the cell fractions with no AFC as determined by ELISpot (432). A previous report noted that parenteral immunisation induced a heterogeneous population of circulating plasma cells containing both terminally differentiated B cells ($CD19/20^{-}$

) and intermediate plasmablasts ($CD19/20^{+}$) (518). However, it has also been reported in humans that plasma cells can survive in secondary lymphoid tissues as well as the bone marrow and that in the secondary tissues the plasma cells maintain CD20 expression (519). This was also shown in a previous study of human plasma cells by Medina *et al* (262) where CD20 expression was highest in the secondary lymphoid tissues (tonsil) reduced in the blood and absent in the bone marrow. Therefore the CD20- and CD20+ populations maybe plasma cell populations with homing potential for either the bone marrow (CD20-) or secondary lymphoid tissues (CD20+).

Furthermore, when PBMCs were sorted on the basis of CD5 expression (following depletion of T cells), immunoglobulin secretion occurred in both the $CD5^{+}$ and $CD5^{-}$ populations. CD5 is the surface marker used to identify the B1a cell population. The frequency of these B cells declines with increasing age dropping from 54% of B cells at 12 months of age to 25% at 24-48 years of age (520). CD5 is also lost during the transition from plasmablast to plasmocyte, further establishing the heterogeneity of the circulating AFC population on day 7 after Pnc7 immunisation in adults (518).

CD5 based sorting also revealed that different phenotypes of plasma cell were involved in responses to protein versus polysaccharide antigens. Most of the diphtheria toxoid specific AFC were in the $CD5^{-}$ population while the B cell response to serotype 4 polysaccharide was mediated mainly by the $CD5^{+}$ population. Earlier studies have looked at responses to tetanus and cholera toxin B-subunit vaccines, where CD5 expression was detected on a small proportion of the IgG and IgA secreting cells, for example; 28% of tetanus toxoid specific IgG AFC express CD5 (518). Together these data suggest that protein specific plasma cells arise from a different B cell subset compared to the polysaccharide specific cells.

In order to re-circulate to the lymph node or bone marrow, plasma cells need to be responsive to locally secreted chemokines. Sorting based on the expression of the chemokine receptors

CXCR4 and CXCR5 revealed that vaccine antigen specific antibody secretion occurred in both the $CXCR4^{+/-}$ and $CXCR5^{+/-}$ fractions. The literature suggests that the expression of chemokine receptors controls the migration of plasmablasts from the medullary cords or B cell follicles to maturation and survival niches in the bone marrow, secondary lymphoid tissues, the mucosa and locations of inflammatory response for the sole purpose of maintaining antibody production (257, 262, 348, 521, 522). The expression of CXCR5 mediates homing to the secondary lymphoid tissues in response to CXCL13 (338, 521) while CXCR4 is upregulated on activated $CD27^{+}$ B cells and is highly expressed on extrafollicular plasmablasts (348, 522). In murine CXCR4 k/o models there was build up of plasma cells in the secondary lymphoid tissues and blood with no migration to the bone marrow. Therefore it was suggested CXCR4 mediates plasmablast homing to the bone marrow in response to bone marrow stromal cell production of CXCL12 (262, 348, 521). Medina *et al* (262) demonstrated increased CXCR4 expression on human plasmablasts in the bone marrow compared to the blood with even lower expression in the tonsils, suggesting a migration pattern from lymph node, through blood to the bone marrow. Thus the data from the study described in this thesis suggests that Pnc7 immunisation induced a plasmablast population that is split between homing to the bone marrow and returning the secondary lymphoid tissues. It is also possible that the phenotypic differences in these plasma cells at day 7 post booster immunisation represent newly generated, vaccine specific plasmablasts versus mature, non antigen specific plasma cells dislodged from bone marrow niches (257, 347). Such that $CD20^{+}$, $CD5^{+}$ and $CXCR5^{+}$, $CXCR4^{+}$ may indicate newly formed plasmablasts migrating to secondary lymphoid tissues or bone marrow to mature into $CD20^{-}$, $CD5^{-}$, $CXCR5^{-}$, $CXCR4^{-}$ mature plasma cells.

7.5 *In vitro* stimulation of peripheral blood B cells affected the frequency of memory B cell derived IgG-AFC.

Enumeration of antigen specific, peripheral blood memory B cells requires *in vitro* stimulation of PBMCs to induce differentiation in to AFC that are then detected by ELISpot. In this thesis PBMCs were stimulated for 5 days using SAC+IL-2 (SI) or SAC+CpG+PWM (SCP). The outcome of the culture systems was quantified by antigen specific ELISpot for the detection of IgG-AFC and B cell proliferation by CFSE dye dilution, at each time point after immunisation (day 0, 6, 7, 15 and 28).

The frequency of total-IgG-AFC detected by ELISpot was significantly higher using SCP than with SI. This is expected as all B cell subsets are stimulated by SCP, while SI only stimulates memory B cells. This difference between the two systems was also true for tetanus toxoid specific IgG-AFC frequency. The proliferation of B cells *in vitro* supported the ELISpot findings in that SI induced proliferation of memory B cells with $CD27^{+}IgG^{+}$ or $CD27^{+}IgD^{lo}$ phenotypes but not with IgD^{hi} or $CD27^{+}IgM^{+}$. While SCP induced proliferation of both memory ($CD27^{+}IgD^{lo}$ and $CD27^{+}IgG^{+}$) and naïve ($CD27^{-}IgD^{hi}$) B cell populations (Chapter 5, figures 5.9-5.12). Therefore it seems that SI induced proliferation and differentiation of switched memory B cells ($CD27^{+}IgG^{+}$ or $CD27^{+}IgD^{lo}$) and SCP induce proliferation and differentiation of switched memory B cells and also IgM memory B cells (CD27+IgM+) and naïve B cells accounting for the higher IgG-AFC frequency in the ELISpot.

SCP stimulation induced more polysaccharide specific IgG-AFC formation in bloods taken at baseline, but in the first seven days after immunisation there was no difference in polysaccharide specific IgG-AFC frequency between SCP and SI stimulation.

From day 15 onwards SCP induced differentiation of more B cells into IgG-AFC than SI did. So it may be that different signals are required for B cell activation depending on the timing after immunisation. The difference in signals required by B cells in the first week compared to the third and fourth weeks after immunisation may reflect differentiation status of the B cells.

Previous studies have shown that *in vitro* stimulation of B cells with SAC only induces proliferation and differentiation of B cells recently activated by specific antigen *in vivo* (437, 438), while IL-2 enhances SAC stimulation of memory B cells (212, 213, 438, 444, 463, 464). The addition of CpG-ODN to the polyclonal stimuli provided appropriate signals for the proliferation of both memory and naïve B cells, although proliferation rate of the naïve B cells was slower (fig.5.12).

Memory B cells constitutively express the intracellular TLR9 (the CpG receptor), while it is low or absent in naïve B cells (228, 351). BCR ligation is needed to signal the upregulation of TLR9 expression by naïve B cells, therefore the proliferative response is delayed compared to memory B cells (228, 470-472).

A recent murine study of TLR9 ligation saw induced differentiation of B1 and MZB cells, but not FO B cells, into mature plasma cells, promoting high amounts of IgM and IgG secretion (299). This might account for the accumulation of marginal zone like ($IgM^{+}IgD^{lo}$) B cells in the SCP culture while mainly IgG^{+} memory cells accumulated in the SI culture.

Following *in vitro* stimulation of PBMCs in this study with SCP there was a greater accumulation of $CD27^{+}$ B cells than with SI, particularly IgG^{+} and $IgM^{+}IgD^{+}$ with most of the IgD^{+} cells being IgD^{lo}.

The secretion of immunoglobulin in culture or as quantified by AFC activity in ELISpots is linked to the cell division number *in vitro* and >4 cell divisions is required following *in vitro* stimulation of PBMCs (387, 416, 417, 446, 451).

The number of memory B cells undergoing >4 cell divisions in the SI system was lower than in the SCP system. However, when the two *in vitro* systems were compared the observed difference in proliferation did not affect the IgG-AFC frequency detected in the ELISpot of cells isolated in the first 2 weeks after immunisation (Chapter 5, fig.5.2). This suggests that antigen specific B cells isolated in the first week after immunisation are already partially differentiated when they enter the *in vitro* systems, thus requiring minimum stimulation to differentiate into plasma cells. It has been suggested that resting FO B cells require BCR cross-linking plus cognate T cell help (299, 315) for full activation and differentiation, but cells recently activated *in vivo* may only require SAC+IL-2 for continued differentiation (437, 438).

From day 15 onwards the SCP system induced greater proliferation and differentiation of B cells into IgG-AFC. Thus the combination of SAC (BCR cross-linking) plus CpG (TLR9) along with PWM (B cell and T cell stimulation) was thought to be optimal to induce proliferation and differentiation of all B cells into IgG secreting cells.

7.6 The responsiveness of B cells to *in vitro* stimulation was altered by increasing age.

The proliferation of memory B cells following *in vitro* stimulation was lower in PBMCs obtained from elderly adults than young adults. In the SCP system the difference between the age groups was significant but not for SI. There was no difference in the proliferation of naïve B cells between the age groups.

The age associated difference in memory B cell proliferation may be accounted for by increased sensitivity of $CD27^+$ B cells to apoptosis in elderly adults compared to young adults while $CD27^-$ B cells do not alter as dramatically (523). However this report was conducted in adults older than 70 years of age. The age difference between the groups in this thesis (mean age of 33 years vs 59 years old) might not be extreme enough to see such a difference in susceptibility of peripheral B

cells to death. As well as increased susceptibility to apoptosis, the percentage of $CD27^+$ B cells declines in the elderly (524). There is also a decline in the numbers of B1 cells with age (520).

In the elderly there were significantly fewer IgM^+IgD^+ B cells proliferating to SI stimulation at baseline (day 0) than was observed for the young adults, but this difference disappeared following a single dose of Pnc7. Since there was no observed age effect on naïve B cell proliferation these data suggest that it is the $CD27^+IgM^+IgD^+$ B cells that are affected in the elderly population. Previous investigators have shown that $CD27^+IgM^+IgD^+$ B cells decrease with age (250, 251, 253) and that *in vivo* proliferation of peripheral B cells (525) and *in vitro* differentiation into plasma cells declines with age (250).

These studies support the proliferation data obtained in this thesis where proliferation of IgM^+IgD^+ B cells in response to SCP stimulation was impaired in the elderly adults compared to the young adults with fewer IgM^+IgD^+ B cells achieving >4 cell divisions in the elderly group.

However, age did not affect the proliferation of IgM^+IgD^+ B cells in response to SI stimulation of PBMCs 28 days after Pnc7 immunisation. This result implies that the B cells stimulated by SI are not affected by increasing age.

This result is also supported by the ELISpot data from this thesis comparing the pneumococcal polysaccharide vaccine with Pnc7. Only the conjugated polysaccharides induced a significant rise in memory B cell frequency as detected by cultured ELISpot. A similar rise in frequency was not seen in response to plain polysaccharides. MZB and B1 cells mediate natural immunity to pneumococcal polysaccharides, so a reduced capacity of these cells to proliferate *in vitro* may explain the lower frequencies of polysaccharide specific memory B cell derived IgG-AFC at baseline in the elderly compared to young adults.

Since IgM^+IgD^+ B cells decrease in frequency with age (123, 250, 251, 253, 520) elderly adult humoral responses may be preferentially mediated by follicular B cells that require T cell help (526). The tetanus and diphtheria IgG-AFC responses obtained in this thesis where similar in

both young and elderly adults and these responses are mediated by FO B cells (123, 305, 502). So this data confirms that the FO B cell pathway is functional in both age groups while the B1 and MZB cells providing natural immunity and extrafollicular responses are reduced or impaired in the elderly group. The increasing susceptibility of the elderly to disease may result from impaired B1 and MZB cells mediated immunity that ordinarily provides rapid production of short lived plasma cells secreting IgM and IgG (123, 315, 527).

7.7 Age affects the frequency and generation of memory B cells in the peripheral blood

Age affects the response to immunisation, but the factors which contribute to the responses are complex. This thesis examined the effect of age on pre-existing memory B cell frequency with specificity for the vaccine polysaccharide and carrier protein antigens. Also determined were the differences in generation of IgG memory following immunisation with Pnc7 in the different age groups.

First, there was no significant difference in the frequency of carrier protein (diphtheria toxoid) specific memory B cells prior to immunisation, but a slightly lower frequency in tetanus toxoid specific memory B cells in the toddler group compared to the young adults on day 7 after immunisation. Therefore age did not affect the thymus dependent response to the protein antigens such as diphtheria toxoid.

Pre-immunisation frequencies of polysaccharide specific memory B cells were lowest in toddlers followed by the elderly adults and young adults had the highest frequencies. Interestingly this age related frequency of polysaccharide specific memory B cells follows what is known about the frequencies of MZB with age (see figure 1.12) (251). MZB are low in frequency and are not fully mature in the <2 year olds, they peak in young adults and wane again after 35yrs of age. B1 cells also wane with age and but are present in the <2 year olds. B1 cells secrete IgM and the ELISA

data for the toddler group show strong IgM responses to all three serotypes tested and there is also evidence of class switching to IgG 56 days after immunisation. The young adults also make good IgM responses to the Pnc7 vaccine pneumococcal serotypes but proportionately more IgG as is seen in secondary responses.

B1a cells secrete natural IgM antibody specific for phosphorylcholine (PCh) residues of pneumococcal CwPS without the need for prior exposure (268). This natural antibody is essential in controlling early infection by blood borne pathogens (268, 286, 426). B1a cells are absent in CD19 knock-out mice and xid mice leaving them vulnerable to overwhelming infection by pneumococci (268, 286, 426). However, B1b cells secrete IgM and some IgG3 in response to the capsular polysaccharides of pneumococci, but require previous exposure to the organism in order to respond. Therefore toddlers may be more susceptible to invasive pneumococcal disease as a result of immature MZB and lack of prior nasopharyngeal carriage to prime the immune system for more rapid IgM and IgG responses by the B1b cells (56, 397, 497, 528). Murine colonisation models have shown that previous colonisation with pneumococcal serotypes enhances IgG responses to subsequent immunisation with pneumococcal conjugate vaccines (497, 528).

Elderly adults lose both the MZB and B1 cells as they age and this may account for increased IPD susceptibility (520). The data reported here have shown that pre-existing memory to pneumococci is low in the elderly but they still make good responses to immunisation possible via the FO B cells pathway.

7.8 Conclusions and future work

The generation of IgG memory in response to immunisation with Pnc7 vaccine is affected by age, and previous priming through either nasopharyngeal carriage or previous immunisation. The rate of IgG memory induction is also dependent on the serotype of the pneumococcus (513).

Prior to immunisation elderly adults and toddlers had low frequencies of IgG memory B cells specific for the capsular polysaccharides. This may be related to the reduced frequencies of MZB in both age groups and also immature status in toddlers. Natural IgG antibody produced by MZB and B1b cells requires previous exposure to polysaccharide antigens and toddlers have experienced fewer episodes of nasopharyngeal carriage than adults. Thus the immune response is dominated by IgM antibody in absence of natural priming. Toddlers had high levels of natural serum IgM, equivalent to that of young adults and levels were highest to serotypes shown by other investigators to be more commonly carried (79). The frequency of B1 and MZB cells decreases with age, thus despite life long exposure to pneumococci the pre-existing IgG memory (using SCP stimulation) detected in elderly adults was lower than in young adults. Both toddlers and elderly adults responded to TD-antigens equally as well as young adults suggesting the FO B cell response was intact in all ages.

In Figure 7.1 I have summarised the picture which has been clarified by these studies showing the development of the B cell response and how this relates to the data discussed in this thesis.

Following immunisation of young adults with either a single of booster dose of pneumococcal conjugate vaccine antigen specific B cells undergo rapid, extrafollicular proliferation and differentiation into short lived plasma cells (fig 7.1, panel A). The extrafollicular response is T cell independent and results mainly in IgM secretion unless priming has occurred via previous exposure to pneumococci or immunisation. The response is rapid and may be mediated by B1a cells, B1b cells, MZB and FO B cells with the resulting, short lived plasma cells appearing in the peripheral blood by day 4 after immunisation.

Panel B of figure 7.1 shows how the *ex vivo* plasma cell data (solid green line) from the young adult studies in this thesis fits with the known extrafollicular kinetics of the B cell response shown in panel A. The magnitude of this extrafollicular IgG response (*ex vivo* plasma cells) was much lower in toddlers than in young adults, probably as a result of fewer priming events prior to

primary immunisation and low frequencies of MZB that are in an immature state. Administration of a second toddler dose of Pnc7 2 months after the first dose did not enhance this IgG plasma cell response, even though serum IgG concentrations and IgG memory B cells increased in frequency. This may be confirmation that the cells involved in the extrafollicular response in toddlers are too few in numbers and are too immature to mount IgG responses to TI-2 antigens with out T cell help. Thus the IgG memory response is likely to result from FO B cell responses following germinal centre development.

Panel A, in figure 7.1 also shows the concurrent development of the germinal centre response with recruitment of TFH cells brought into close contact with B cells by antigen expressing FDC. The germinal centre B cells undergo further rounds of proliferation (purple cells) at which point class switching and somatic hypermutation of antibody variable genes occurs, increasing antibody avidity to specific antigen.

The cells which exit the germinal centre include plasmablasts that reside in the peripheral lymphoid tissues secreting antibody for prolonged periods, long-lived plasma cells homing to the bone marrow and memory B cells that re-circulate throughout the peripheral blood and lymph.

Stimulation of PBMCs with SI enabled detection of IgG-AFC for up to 1 month after immunisation of young adults (red dotted line in panel B, fig 7.1). Prior to immunisation there was limited detection of IgG-AFC via this system in young and elderly adults and none in toddlers. *In vivo* exposure of B cells to specific antigen is required for continued proliferation and differentiation of these B cells during *in vitro* stimulation with SI. IgG-AFC derived from B cells in this culture system were only detected at significant levels during the first two weeks after immunisation.

The germinal centre response begins to involute by day 22, disappearing by day 28 (panel A, fig 7.1). The SI induced IgG-AFC frequency waned with similar timing to the germinal centre reaction, suggesting that these cells were products of IgG secreting germinal centre B cells.

Future work will be undertaken to measure the avidity of IgG secreted *in vitro* at each time point to confirm whether the B cells were high avidity antibody secreting germinal centre plasma cells or extrafollicular B cells.

IgG-AFC detected following SCP stimulation of B cells *in vitro* peaked at day 15 (blue dotted line in panel B). This time point coincides with the peak in germinal centre activity with cells in various states of differentiation exiting in to the peripheral blood. SCP provides the signals for activation of B cells in all states of activation. B cells exiting the germinal centre include cells that have not recently encountered specific antigen which may explain the difference in IgG-AFC frequency between the SCP and SI system (panel B).

Following immunisation the magnitude of the IgG memory B cell response in the young and elderly adults was equivalent, but in toddlers it was much lower. Serum antibody responses to primary immunisation in toddlers were dominated by IgM and the rate of generation of IgG memory was serotype dependent with serotype 4 switching to IgG more rapidly in toddlers while in adults serotype 23F switched more rapidly (a scheme of antibody responses to immunisation of young adults is shown in fig.7.1, panel C). This difference in IgG memory generation was most likely a result of less priming via nasopharyngeal carriage and in support of this a second dose of Pnc7 in toddlers produced IgG memory responses similar to those in adults after a single dose.

Further work is planned on depletion of B cell subsets to identify the cells at each time point that are involved in the IgG and IgM production in response to SAC+IL-2 or SAC+CpG+PWM. Stimulation of B cells with specific polysaccharide along with co-stimulation by CpG and/or PWM may also help to identify the time point at which antigen activated B cells appear and disappear from the periphery. This has been studied with plain polysaccharide vaccines (109), but not conjugated polysaccharides vaccines. It might be expected that antigen specific memory cells would be present in peripheral blood for longer if germinal centre formation had been induced by the conjugated rather than the plain polysaccharide vaccine. Identification of antigen

specific B cells using intracellular labelling of antigen of antigen specific plasma cells with fluorescent tagged polysaccharides will also be utilised to investigate this question.

In conclusion, when determining the B cell or serum antibody response to glycoconjugate vaccines it is important to measure the IgG and IgM response, particularly in the very young. IgM is the first line of defence against blood borne pathogens and low frequencies of the cells responsible for secretion of IgM coincides with the age at which invasive disease peaks. Enhancing IgM memory may be important for protection and can be replaced by generation of a high avidity IgG response to enhance protection in the very young and elderly through immunisation.

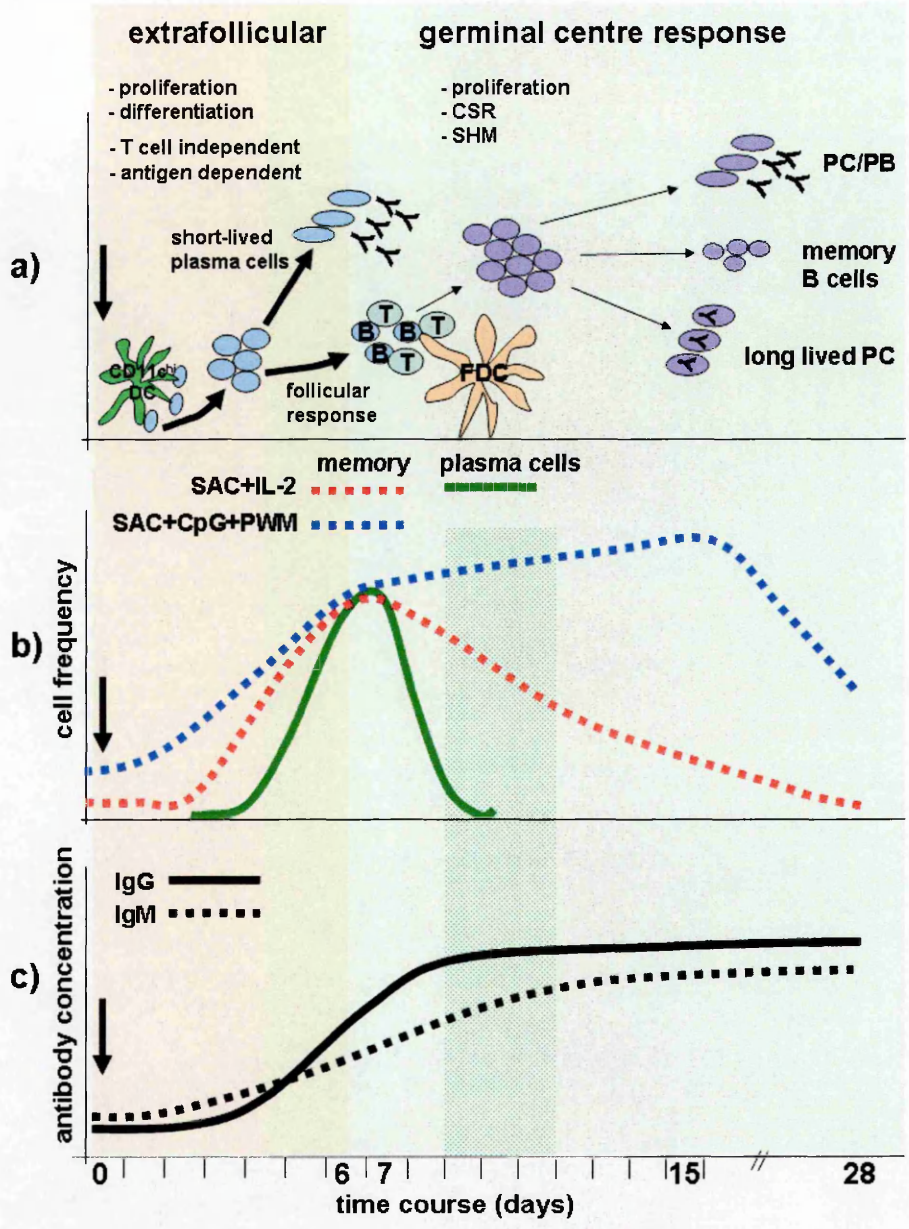


Figure 7.1 Schematic of the B cell response following immunisation of young adults with Pnc7 vaccine.

The orange background represents the extrafollicular response time and the green background the germinal centre response with cross over at days 5-7.

Panel a) The B cell response following immunisation with the extrafollicular response represented by blue cells and the later germinal centre response as purple cells. PC=plasma cell, PB=plasmablast, CSR = class switch recombination, SHM, somatic hypermutation, B=B cell, T=T cell, Y=antibody, DC= dendritic cell, FDC= follicular dendritic cell. Panel b) ELISpot IgG-AFC kinetics (the darker green, hatched area represents the initial time points of the original toddler vaccine study (Chapter 3). Panel c) Serum IgG and IgM responses to the polysaccharide antigens during the first month after immunisation of adults with the Pnc7 vaccine. The black arrows show the Pnc7 immunisation day.

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